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(54) Title: PRODUCTION AND USE OF DOPAMINERGIC CELLS TO TREAT DOPAMINERGIC DEFICIENCIES			
(57) Abstract			
<p>Differentiated neuronal cells suitable for transplantation in individuals with a dopamine deficiency are derived from progenitor cells. The progenitor cells are treated with at least one inducing agent such as retinoic acid for a time period sufficient to optimize expression of tyrosine hydroxylase. The cells intended for transplantation are optionally treated with a lithium salt to enhance bcl-2 production and survival. Optionally, the progenitor cells may be co-cultured with Sertoli cells, astrocytes, glial cells, accessory cells or a combination thereof. The transplantation-ready cells are isolated and harvested. The resulting neuronal cells are highly purified and have a phenotype optimized for a dopaminergic deficiency, such as Parkinson's Disease.</p>			

DescriptionProduction and Use of Dopaminergic Cells to Treat Dopaminergic Deficiencies5    Technical Field

The present invention is in the field of tissue engineering/transplantation and more particularly dopaminergic cells, their production and use in transplantation.

Background Art

10      Dopaminergic neurons are those which synthesize and use dopamine (DA) as a neurotransmitter. Dopaminergic neurons are found in a number of areas of the brain, including the nigrostriatal, mesolimbic, mesocortical and tubero-hypophysial systems. The rate-limiting step in dopamine synthesis is catalysis of tyrosine by tyrosine hydroxylase (TH). Dopamine is stored in synaptic vesicles. Dopamine is released from presynaptic vesicles by exocytosis.

15      Dopamine acts on as many as five classes of receptors. Dopamine is recycled by reuptake and/or degradation by monoamine oxidase B (MAO-B) (RK Murray, Ch. 64. The Biochemical Basis of Some Neuropsychiatric Disorders. In: Harper's Biochemistry, ed. by Murray, et al. 24th ed., Appleton & Lange, Stamford, CT, 1996, pp. 794-814).

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a loss of 20 dopaminergic cells from the substantia nigra par compacta, resulting in decreased dopaminergic input to the striatum. The hallmark motor symptoms include tremor, rigidity, bradykinesia, and instability. In spite of a host of approved pharmacological and surgical treatments, existing therapies for PD are only partial and palliative. Levodopa (L-dopa) the gold standard pharmacological treatment to restore DA, is plagued by decreased efficacy and increased side effects over time. Adjunct treatment with DA agonists is frequently necessary; however, 25 recently approved DA agonists with greater receptor subtype specificity may provide only incremental clinical benefit. Catechol-O-methyltransferase (COMT) inhibitors to slow DA metabolism soon will be joining monoamine oxidase (MAO) inhibitors.

To replace the missing cells, there is a renaissance of neurosurgical treatments for PD. 30 After all pharmacological treatments have failed, surgical procedures including pallidotomy, thalamotomy and deep electrical stimulation may be considered. Nevertheless, for almost one million individuals in the US afflicted by PD, a reliable long-term treatment to halt disease progression remains elusive.

Schizophrenia is often treated by neuroleptic drugs which decrease the amount of 35 dopamine activity in mesolimbic dopaminergic neurons. "Positive symptoms" (e.g., hallucinations, delusions, bizarre behavior) have been associated with excess dopamine

activity in the mesolimbic neurons. "Negative symptoms" of schizophrenia (e.g., social withdrawal, emotional blunting, and catatonia) may be associated with low dopamine activity in the prefrontal cortex. Since prefrontal dopaminergic neurons may normally inhibit the activity of subcortical dopamine neurons, a lowering of dopamine in the prefrontal area could lead to the elevated dopaminergic activity in the subcortical neurons.

5        Progressive Supranuclear Palsy (Steele-Richardson-Olszewski Syndrome) is due to a loss of neurons and gliosis in the tectum and tegmentum of the midbrain, the subthalamic nuclei of Luys, the vestibular nuclei, and to some extent the ocular nuclei. Some symptoms are shared with Parkinson's disease, including rigidity of the neck and other trunk muscles and occasional 10      sensitivity to L-dopa.

A rare form of torsion dystonia is dramatically L-dopa-responsive. Starting in childhood, the dystonia first affects gait. Most individuals later develop parkinsonism. Some focal dystonias also are reported to be L-dopa responsive.

15       In a neurodegenerative disorder associated with autonomic failure (i.e., Shy-Drager Syndrome), positron emission tomography has shown decreased uptake of dopamine derivatives in the putamen and caudate, probably reflecting a loss of nigrostriatal dopaminergic neurons. Current treatment is symptomatic. The parkinsonian symptoms may be helped by L-dopa or other dopaminergic drugs, but later most patients become refractory to these drugs.

20       Depression is associated with heterogeneous dysregulations of the biogenic amines. Although norepinephrine and serotonin have been most implicated in the pathophysiology, dopamine also may play a role in depression. Dopamine may be reduced in depression and increased in mania. Drugs that reduce dopamine concentrations (e.g., reserpine) and diseases that reduce dopamine concentrations (e.g., Parkinson's disease) are associated with depressive symptoms. Also, drugs that increase dopamine concentrations (e.g., tyrosine, amphetamine and 25      bupropion) reduce the symptoms of depression. Two recent theories regarding dopamine and depression are that the mesolimbic dopamine pathway may be dysfunction in depression and that the dopamine type 1 (D1) receptor may be hypoactive in depression (Ch.9. Mood Disorders, in: CONCISE TEXTBOOK OF CLINICAL PSYCHIATRY. Ed. by HI Kaplan and BJ Sadock. Williams & Wilkins, Baltimore, MD, 1996, pp. 159-188).

30       MAO inhibitors also are the drugs of choice in agoraphobia (irrational fear of being alone or in public places) and panic disorder. There also is growing evidence that MAO inhibitors are effective in the treatment of some anxiety disorders, particularly mixed depressed and anxious states.

The search for a continuous, stable, regulated, site-specific source of DA delivery has

turned to tissue transplantation, cell therapy and genetic engineering, with the ultimate goal of finding an effective treatment to halt or reverse disease progression.

Human fetal mesencephalic tissue transplants are the most extensively studied procedure. They have demonstrated therapeutic potential in animal models of PD and in Parkinson's disease patients. Fetal tissue transplants have been performed in the clinic for over a decade on more than 200 patients throughout the world with positive outcomes (Kordower JH, Goetz CG, Freeman TB, Olanow CW. *Experimental Neurology* 144:41-46, 1997). Grafts survive, form synaptic connections, and improve motor function in many patients. However, ethical, moral and technical constraints limit the widespread use of human fetal tissue. Future progress in the field of neural transplantation will depend largely on the development of alternative sources of cells.

Xenotransplantation, the use of cells from different species, is a viable approach to circumventing the limitations associated with human fetal neural transplantation (Galpern WR, Burns LH, Deacon TW, Dinsmore J, Isacson O. *Experimental Neurology* 140:1-13, 1996). A phase I clinical trial sponsored by Diacrin, Inc., is evaluating transplants of porcine cells harvested from the midbrains of pig fetuses. Another technique, developed by Cytotherapeutics, Inc., uses encapsulated xenografts of rat PC12 cells that secrete dopamine. A semipermeable polymer membrane allows diffusion of the small therapeutic molecules but prevents diffusion of the larger immunogenic molecules. Whether the release of dopamine from encapsulated sources will be sufficient to restore optimal DA levels in PD patients remains to be determined. Although cells derived from animals are potential candidates for human neural transplantation, they carry the risks of transferring intrinsic pathogens, creating novel infectious agents, or eliciting deleterious immune responses (Isacson O, Breakefield X. *Nature Medicine* 3:964-969, 1997).

Cell therapy for PD, which is still at the experimental stage, is theoretically capable of reversing neurotransmitter deficiencies, halting neural degeneration, and repairing neural damage. Many types of cells (e.g., rat fibroblasts) have successfully been transfected *ex vivo* with, for example, the human tyrosine hydroxylase (TH) gene to serve as a local source of dopaminergic factors (Raymon HK, Thode S, Gage FH. *Experimental Neurology* 144:82-91, 1997). Concerns about long-term stable gene expression, tumor formation, and pathogen delivery need to be resolved.

*In vivo* gene therapy is possible using direct insertion of genes into brain cells via viral vectors (herpes simplex virus, adenovirus, adeno-associated virus, or lentivirus). Genetically modified vectors encoding genes such as TH or glial-derived neurotrophic factor models of PD.

However, the extent of gene expression, long-term efficacy, and cytopathogenicity associated with viral vectors is unknown.

Growth factors such as GDNF and brain-derived neurotrophic factor (BDNF) can be delivered alone or in combination with tissue transplants to provide trophic support and protect dopaminergic cells (Rosenblad C, Matinez-Serrano, Bjorklund A. *Neuroscience* 75:979-985, 1996). The long-term benefits and risks are unknown. Delivery is problematic, but novel approaches via injection directly into the brain, a Medtronic device, encapsulated cells, and genetically engineered cells are under investigation.

Recent research has focused on adapting NT2 or hNT cells for treatment of Parkinson's Disease (Iacobitti and Stull, *NeuroReport* 8:1471-74, 1997). Both newly differentiating human neurons (hNT cells) and the undifferentiated precursors (NT2 cells) were treated with a variety of factors. In hNT but not NT2 cells, TH expression was induced by aFGF and co-activators (DA, TPA or IBMX/forskolin). A moderate number of hNT cells were said to have been induced; however, data were not provided. hNT cells were not induced with only one of aFGF or a co-activator. The induced hNT cells aggregated and hampered cell counting. Nevertheless, it appeared that, with increasing time in culture, more hNT cells expressed TH. After five days, 565 out of  $10^5$  plated hNT cells or less than 1%.

Lithium, the primary treatment for mania and bipolar affective disorder, has been reported to significantly influence the activity of signaling systems. Using PC12 cells as a model system, Li and Jope (*J Neurochem* 65:2500-08, 1995) studied the NGF-induced expression of several signal transduction proteins, including subtypes of G proteins, protein kinase C and phospholipase C and its modulation by lithium. Their results demonstrated that lithium, at a therapeutic concentration (1mM), modulates the level of signal transduction proteins. Several studies have indicated that the activation of TH by intracellular calcium ion could be mediated by calcium/calmodulin-dependent protein kinase (for review, see Masserano et al., "The Role of TH in the Regulation of Catecholamine Synthesis." In handbook of experimental pharmacology. Vol 90/II Catecholamines, Ed. by Trendelenburg and Weiner, Springer Verlag, Berlin, 1990, pp 427-69). However, controversial results have been obtained when lithium has been studied in relation to the brain content of catecholamines. Both decreased synthesis of dopamine (Friedman and Gershon, *Nature* 243:520-21, 1973) and up-regulated TH activity (Segal et al., *Nature* 254:58-59, 1975) have been reported after lithium treatment, perhaps due to the complexity of the brain tissue. On the other hand, increased synthesis and secretion of catecholamines and protein kinase C activity was demonstrated (Terao et al., *Biol Psychiatry* 31:1038-49, 1992) when lithium was applied on cultured adrenal

medullary cells.

In summary, there is substantial evidence in both animal models and human patients that neural transplantation is a scientifically feasible and clinically promising approach to the treatment of PD. Nevertheless, alternative cell sources and novel strategies are needed to circumvent the numerous ethical and technical constraints that now limit the widespread use of neural transplantation.

According to Anton et al. (Anton R, et al. Exp Neurol 127:207-218, 1994), the ideal cell for a CNS transplant system should meet the following criteria: It should be of human CNS origin, capable of growth cessation and differentiation, clonal and defined, transfectable and selectable, immunologically inert, capable of long-term survival following implantation, non-tumorigenic, functional and integrated into the host brain, of consistent quality, and readily available.

#### Disclosure of Invention

Dopaminergic neuronal cells suitable for transplantation in dopaminergic deficiency are derived from progenitor cells as follows. The progenitor cells are treated with retinoic acid for a time period sufficient to optimize expression of tyrosine hydroxylase. The optimized neuronal cells being further treated with at least one lithium salt or a combination thereof. The transplantation-ready cells are selected with trypsin and so harvested. The resulting neuronal cells are highly purified and have a phenotype optimized for at least one neurodegenerative disease, such as Parkinson's Disease. Optionally, the progenitor cells also are cultured with Sertoli cells, astrocytes, or glial cells.

#### Brief Description of Drawings

Figure 1 is a tyrosine hydroxylase Western blot of NT cells at different stages of maturation in the hNT-Neuron process. It was developed with anti-TH monoclonal antibody and biotin-strepavidin alkaline phosphatase system. The following lanes contain: 1) 500 pg TH rat standard, 2)  $1 \times 10^6$  hNT-Neurons positive control, 3)  $1 \times 10^6$  Replate-I Neurons, 4)  $1 \times 10^6$  Replate-I Accessory cells, 5)  $1 \times 10^6$  NT2/D1 Precursor cells. Lanes 6-8 contain  $1 \times 10^6$  Replate-II neurons each harvested after 1 week (lane 6), 2 weeks (lane 7), and 3 weeks (lane 8) in extended culture.

Figure 2 is a tyrosine hydroxylase Western blot comparing different maturation conditions for the hNT-Neurons. It was developed with anti-TH monoclonal antibody and biotin-strepavidin alkaline phosphatase system. NT2/D1 cells were induced with RetA for 6

weeks and processed as Replate-I or Replate-II cultures in mitotic inhibitors for 1 week. Then the cultures were allowed to mature in conditioned media for 1 day (1 week replate), 1 week (2 week replate), or 2 weeks (3 weeks replate). Pure hNT-Neurons were harvested from the mature replate cultures and cell extracts corresponding to  $1 \times 10^6$  cells were loaded in the following lanes. Lanes 1-3 show the results for the Extended Replate-I Neurons which were matured for 1 week (lane 1), 2 weeks (lane 2), and 3 weeks (lane 3). Lanes 5 and 6 have Replate-II Neurons which were matured for 1 week and 2 weeks, respectively. Lanes 4 and 7 contain  $1 \times 10^6$  hNT-Neurons positive control.

Figure 3 is a TH Western Blot showing the time course of RetA Induction. It was developed with anti-TH monoclonal antibody and biotin-strepavidin alkaline phosphatase system. The NT2/D1 cells were induced with RetA for 4, 5, or 6 weeks, and after induction Replate-I cultures were maintained in mitotic inhibitors for either 1 week (Lanes 2-4) or a total of 2 weeks matured (Lanes 5-7). Purified hNT-Neurons were harvested from each sample and cell extracts corresponding to  $1 \times 10^6$  cells were loaded in the following lanes. The 1 week Replate-I: 2) 4w-RetA, 3) 5w- RetA, 4) 6w-RetA. The 2 weeks matured Replate I: 5) 4w-RetA, 6) 5w-RetA, 7) 6w-RetA. 8) hNT positive control, and rat TH standard was in lanes 1 (500pg) and 9 (5ng).

Figure 4 is a bar graph showing the effects of different doses of lithium chloride on TH expression in cultured hNT neurons.

Figures 5a -5d are photomicrographs of a representative culture of hNT cells treated with lithium chloride for four days and immunostained with antibodies to tyrosine hydroxylase. Figures 5a and 5b show hNT cells cultured with 1.0 mM concentration of lithium chloride. Figures 5c and 5d represent cultures of hNT cells treated with 3.0 mM concentration of lithium chloride.

Figures 6a through 6c are photomicrographs illustrating the effects of 4 weeks of RetA and 5 days of LiCl on hNT cells on frequency of TH-expressing cells (6a), TH and PI staining (6b) and bcl-2 expression.

Figure 7 is a bar graph showing the effects of different doses of lithium chloride on Bcl-2 expression in cultured hNT neurons.

Figures 8a and 8b are photomicrographs comparing cytochrome oxidase activity in 4-week RetA-treated control hNT cells (8a) and LiCl-treated hNT cells (8b).

Figures 9a, 9b, 9c, 9d, and 9e are photomicrographs of the distribution, morphological appearance and phenotype of hNT neurons 5days in control culture and lithium-treated cultures. (9a) phase contrast low-magnification photomicrographs showing the distribution of hNT

neurons in control/untreated cultures. (9b) higher magnification photomicrograph demonstrating that virtually all cultured hNT cells are immunoreactive for GAP43. (9c) and (9d) are low-magnification phase contrast photomicrographs of hNT neurons treated with 1.0mM (9c) and 3.0mM (9d) concentration of lithium chloride. (9e) Morphological appearance of GAP-43-labeled hNT neurons treated with 3.0 mM lithium chloride.

5 Figures 10a, 10b, 10c, 10d, and 10e are photomicrographs that show the effect of lithium chloride on TH expression in hNT neurons cultured for 5 days.

Figures 11a, 11b, 11c, 11d, and 11e are photomicrographs of representative control and lithium-treated hNT neurons cultured for 5 days and immunostained for tyrosine hydroxylase.

10 (11a) control culture of hNT neurons reveals few TH-positive cells. (11b) and (11c) show hNT cells cultured with 1.0mM (11b) and 3.0 mM (11c) lithium chloride. (11d) and (11e) show the representative morphology of TH-positive hNT cells treated with 1.0 mM (11d) and 3.0 mM (11e) of lithium chloride.

Figure 12 is a table which shows the effect of lithium chloride on soma size (12a) and neurite growth (12b) of hNT neurons cultured for 5 days. \* denotes significant difference (p<0.01) compared to control.

20 Figure 13 represents a process for LiCl Induction of DA neurons. The levels of TH expressed are shown in Figure 13 (DA-Neuron Co-Culture). Lane 1 is 500 pg TH, lane 2 if fresh DA-Neurons bulk harvested. Lane 3 shows the results after 1-week co-culture with polylysine, TM-4 cells are in lane 4 and rat glial cells are in lane 5.

~~X~~ Figure 14 represents the production of LiCl Induced DA-Neurons. The enhanced expression of TH in the 4-week DA-Neurons was found to be optimal in small-scale cultures after replating the neurons in serum free media containing 1 mM LiCl for 5 to 7 days. Figure 14 shows the results for 500 pg TH (Fig. 14 lane 1); control DA neurons in DF-5/Inh, 7 days (Fig. 14 lane 2); DA neurons in DF-5/Inh/ 1mM LiCl, 7 days (Fig. 14 lane 3); DA neurons in DF-5/Inh/ 1mM LiCl, 3 days (Fig. 14 lane 4); DA neurons in DF-5/1mM LiCl, 3 days (Fig. 14 lane 5); DA neurons in DF-5/ 1mM LiCl, 3 days (Fig. 14 lane 6); and DA neurons in DF/ 1% ITS/ 1mM LiCl, 3 days (Fig. 14 lane 7).

### 30 Modes for Carrying Out the Invention

The six criteria for transplantable cells summarized above are met by hNT-Neurons. In addition, hNT-neurons surprisingly were able to be optimized for stable TH production similar to that seen with primary mesencephalic cells. TH is vital because it performs the rate-limiting step in production of dopamine. These optimized PD-hNT neurons have improved

dopaminergic properties arising from manipulating the hNT-neuron natural capabilities. These procedures eliminated the need to transfect the cells with exogenous gene constructs.

5 hNT-Neurons have the potential to overcome many of the limitations associated with human fetal tissue transplantation, including poor graft survival (5-10%), high tissue variability, and low degree of host re-innervation. hNT-Neurons demonstrate excellent graft survival and behavioral improvements in animal models of CNS disorders. There are preliminary data suggesting that hNT-Neurons may have immunosuppressive properties. Thus, long-term, systemic immunosuppression may not be necessary in humans.

Furthermore, hNT-Neurons are human cells derived from the human teratocarcinoma 10 NT2/D1 cell line through induction with RetA treatment (Andrews, PW, Damjanov J, Simon D, Banting G, Carlin C, Dracopoli NC, Fogh J. Lab Invest 50:147-162, 1984). During the 6-week retinoic acid induction period, NT2/D1 cells, which share many characteristics of neuroepithelial precursor cells, undergo significant changes resulting in the loss of neuroepithelial markers and the appearance of neuronal markers (Pleasure SJ, Page C, Lee VM. J Neurosci 12:1802-1815, 15 1992; Lee VM, McGrogan M, Lernhardt W, Huvar A. Strategies in Molecular Biol 7:28-31, 1994). Several enrichment steps result in the production of >99% pure populations of hNT-Neurons that are terminally differentiated (Andrews et al, *ibid.*). They display process outgrowth and establish functional synapses. Thus, mature hNT-Neurons do not divide, they maintain a neuronal phenotype, and they appear to be virtually indistinguishable from terminally 20 differentiated, post-mitotic, embryonic neurons (Pleasure S J, Lee VM. J Neurosci Res 35:585-602, 1993).

Finally, we demonstrate in the studies disclosed herein that the phenotype of the hNT cells can be altered by certain culture conditions - without transfection of foreign genes - to consistently produce the levels of DA that could treat abnormal dopaminergic conditions.

25

#### Definitions:

A dopaminergic deficiency is a condition in which there is a shortage of dopamine. The 30 dopaminergic deficiency may have a variety of causes, including, but not limited to, under-production by dopaminergic neurons, deficit of dopaminergic neurons, or insensitivity of dopaminergic neurons to dopamine. Examples of such conditions include, but are not limited to, Parkinson's disease, schizophrenia, progressive supranuclear palsy (Steele-Richardson-Olszewski Syndrome), and a Dopa-responsive form of torsion dystonia.

"Beneficial effect" is an observable improvement over the baseline clinically observable signs and symptoms. For example, a beneficial effect can include improvements in graft

survival, improvements in one or more of the signs and symptoms associated with a dopaminergic deficiency, such as movement or mood.

“Mammal” includes humans and other mammals that would reasonably benefit from treatment, including pets such as dogs and cats.

5 “NT2/D1 precursor cells” as used herein refers to a special cell line available from Layton Bioscience, Inc. (Atherton, CA). This cell line has been developed from a previously described human teratocarcinoma cell line (termed Ntera2/clone DI or NT2 cells) (Andrews et al. Lab. Invest. 50:147-162, 1981). These cells are precursors for LBS-Neurons™ human neuronal cells. NT2/D1 cells are unique among other teratocarcinoma cell lines because these 10 cells act like progenitor cells whose progeny are restricted to the neuronal lineage (Andrews, ibid.)

15 “LBS-Neurons™ human neuronal cells” as used herein refers to the special neuronal cell line disclosed in U.S. Patent No. 5,175,103 to Lee et al. Briefly, NT2/D1 precursor cells are induced to differentiate into neurons by administration of 10 (M RetA which is replenished three times weekly for 6 weeks, after which the cells are replated with special manipulations to become more than 99% pure hNT-Neurons. These are the cells which are used in the subsequent experiments. Alternately, for human use, there is a cell line manufactured without antibiotics (used in the research grade LBS-Neurons and under good manufacturing practices (GMP) which is termed LBS NEURONS human neuronal cells (Layton Bioscience, Inc.).

20 “Inducing agent” includes, but is not limited to compound which have the same effect in causing NT2/D1 precursor cells to differentiate into hNT neurons, one example of which is retinoic acid. Thus, an inducing agent includes not only retinoic acid in any of its isomers and trans/cis forms, but also similarly active compounds.

25 “Immunosuppressant” as used herein is a substance which prevents or attenuates immunologic phenomena. For example, such immunologic phenomena include inflammation, autoimmunity, GVHD and graft rejection. Examples of current immunosuppressants include but are not limited to cyclosporine A, cyclophosphamide, prednisone and tacrolimus (FK506). Optionally, an immunosuppressant can be administered at the time of the transplant. One regimen calls for administering the immunosuppressant for two days before and on the day of 30 transplantation.

“Vehicle” is a biologically compatible solution, such as 0.9% normal saline and the like, which is used to suspend and inject the dopaminergic cells into mammals. This definition also includes any gel which firms at body temperature and is biodegradable.

As used herein, the term “sample” is meant to refer to one or more treated cells. In

preferred embodiments, a sample contains a plurality of cells. According to the present invention, a sample of treated cells is implanted into either a non-human mammal or a human.

By "lithium" is meant generally a lithium salt, wherein the anion includes but is not limited to chloride, bromide, carbonate, citrate, or other biologically compatible monovalent anion. In particular, lithium chloride (LiCl) has been used in many of the examples disclosed below.

"Therapeutic agent" as used herein means the transplanted cells themselves or chemical entities secreted by these cells. Examples of chemical entities secreted by the cells include, but are not limited to dopamine, other neurotransmitters, proteins and hormones.

10

#### Examples

##### **Example 1. Neuron culture techniques**

The production of hNT-Neurons is an 8-10 week process. All cell culture work is performed in T-flasks. The neurons are induced from NT-2/D1 cells following exposure to growth media containing 10 µM RetA for 4-6 weeks. Cells are harvested using trypsin and replated at reduced density. Replate I cultures are maintained in growth media for 2 days and then selectively harvested with trypsin to give an enriched neuron population and replated (replate II) into flasks. Both extended replate I and replate II cultures are treated with mitotic inhibitors for 7 days. The cells are maintained in neuron-conditioned media and allowed to mature in culture. Neurons constitute approximately 10% of the cell population; 90% are non-neuronal accessory cells. Neurons are post-mitotic and no longer capable of dividing, whereas the accessory cells are mitotically inhibited by the addition of cytosine arabinoside (Ara-C) and fluorodeoxyuridine (FUDR) to culture medium. Neurons, which are loosely attached to the surface of the cell bed, are selectively harvested by brief trypsin treatment. Harvest results in a purified bulk product of >95% neurons.

##### **Example 2. RT-PCR for RH and D<sub>1</sub> and D<sub>2</sub> Receptors**

Reverse transcript PCR (RT-PCR) was used to detect the presence of endogenous and vector DNA and transcribed mRNA for enzymes such as TH. Messenger RNA and cDNA were prepared using commercially available reagents and kits. PCR primers, if not already at hand, were designed using the MacVector software package to minimize primer self annealing and PCR artifacts. PCR was performed using standard parameters (Innis MA, Gelfand DH, Sninsky JJ, White TJ. PCR Protocols. Academic Press, 1990) with commercially available enzymes and TAQ polymerase according to manufacturer's recommendations.

The expected TH band was present in the sample of immature hNT-Neurons which were harvested after 6 weeks of RetA, as well as in the sample of mature purified hNT-Neurons that were aged in culture for 5 weeks after mitotic inhibitor treatment. The TH band was not detected for either the uninduced NT2 precursor cells or the 24hr RetA-induced cultures. These results are consistent with other observations that TH is not expressed in the precursor cells or the NT2 cells early in the induction period (personal communication, Virginia Lee). TH begins to appear only after several weeks of RetA induction as the neurons develop, and continues during the differentiation of hNT-Neurons. Similar results were obtained for the PCR analysis of the dopamine D1 and D2 receptor expression.

10

**Example 3. Western blot assay for TH Expression**

Protein samples and cell extracts were denatured in SDS sample buffer containing  $\beta$ -mercaptoethanol and electrophoresed on a Laemmli SDS polyacrylamide gel which separates the proteins by size. The proteins were then transferred to a nitrocellulose membrane by electroblotting and the specific protein of interest was immunodetected by reacting to a primary antibody which is developed using the appropriate secondary antibody enzyme conjugate system.

Samples were prepared by harvesting cells with trypsin/EDTA solution, or by scraping, and resuspending in media. An aliquot of cells was taken for viable cell count, and total viable cells were determined. The remaining cells were centrifuged and washed, and the resulting cell pellet were quick-frozen and stored at -80°C. Samples were thawed and lysed in cold RIPA buffer at a concentration of  $10^6$  cells in 10 $\mu$ l of buffer, an equal volume of 2X SDS reducing sample buffer added, and heated in boiling water bath for 5 min and then placed on ice.

Samples of 20 $\mu$ l, equivalent to  $10^6$  cells per lane, were loaded on SDS polyacrylamide gels. The TH protein standard (STI, Catalog No. P-20233) at 500pg/ sample and the prestained protein molecular weight marker samples were prepared in SDS reducing sample buffer. The gel was electrophoresed until protein markers were well separated and bromophenyl blue dye had run off the bottom of the gel. The protein samples were transferred from the gel and immobilized on a nitrocellulose membrane by electroblotting in a Western transfer chamber. The Western blot was blocked overnight in PBS containing 2% dried milk and incubated with anti-TH monoclonal (Boehringer-Mannheim clone 2/40/15) in PBS-Tween containing 1.0% BSA. The blot was incubated at room temperature with biotinylated goat anti-mouse secondary antibody (1:1000) and then with Streptavidin-Alkaline phosphatase conjugate (1:2000). The blot was washed in PBS-Tween and developed using the insoluble alkaline phosphatase substrate

BCIP/NTB (Sigma). The TH specific bands are visualized within 1-2min. and appeared in the 55 to 60Kd size range. The sensitivity of the assay was determined to be approximately 50pg/lane using serial dilutions of the TH-protein standard (STI) ranging from 5ng to 20pg.

TH levels confirmed and quantified by Western blot assay. In order to confirm and 5 quantitate the levels of TH expressed in different samples, a Western blot assay was developed. Several monoclonal antibodies were evaluated for detection of TH on blots containing human cell extracts and rat TH standard (STI). The anti-TH monoclonal 2/40/15 (Boehringer Mannheim) was found to detect reproducibly 50 to 100 pg of TH/lane. Initial estimates of the amount of TH protein expressed were determined using the Western blot assay and purified rat 10 TH (STI) as standard. Analyzed samples of hNT-Neurons contained a range of cells from  $2 \times 10^6$  to  $2 \times 10^5$  per lane. The intensity of the TH-specific band was compared to samples containing 500 pg to 50 pg of the TH standard. The results of this analysis indicated that approximately 200-500 pg of TH protein are present in extracts containing  $10^6$  hNT-Neurons. A similar 15 analysis was performed using human mesencephalic samples ranging from  $10^6$  to  $10^5$  per lane. Equivalent levels (200-500 pg/ $10^6$  cells) were detected in the hNT-Neurons compared to the human mesencephalic tissue.

The commercial process of producing hNT-Neurons involves inducing the NT2/D1 20 precursor cells with RetA for 6 weeks. The resulting culture contains 10% neurons and 90% non-neuronal accessory cells, which are next mitotically inhibited. The hNT-Neurons are selectively harvested, leaving the accessory cells behind. The levels of TH expressed in hNT- 25 Neurons, NT-accessory cells, and NT2 precursor cells were compared by Western blot (Figure 1). Only the sample of hNT-Neurons (lane 3) gave the expected TH band that migrated at 55-60 kd, and no TH-specific bands were detected in the accessory cell sample (lane 4) or NT2 precursor cell sample (lane 5).

25

#### **Example 4. Preparations for Immunohistochemical staining**

Cells were fixed with 4% paraformaldehyde in 0.1 M NaPO<sub>4</sub>, pH 7.4 and washed with phosphate buffered saline (PBS). The cells were incubated first in PBS containing 1% serum and 0.1% Triton X-100 for 30 min at room temperature and then overnight in the same solution 30 containing the primary antibody. The cells were washed in PBS containing 1% serum and 0.1% Triton X-100, and then incubated with the biotinylated secondary antibody. Cells were washed as described and placed in 1:500 Strep Avidin HRP for 2 h. Preparations were developed with DAB (following manufacturer's instructions). Photomicrographs were taken, and immunoreactivity of dopaminergic neurons assessed as described below.

**Example 5. Enhancing Dopaminergic Properties of hNT-Neurons**

A series of studies evaluated the dopaminergic potential of other neuronal precursor cell lines, the optimal time for RetA induction of TH during the differentiation of the NT-Neurons, 5 and the process for stabilization of TH during replate purification.

**5.A. Optimal clone selection**

Other neuronal precursor cell lines were evaluated for their potential to produce neurons with dopaminergic properties. The 10 sister clones to the NT2/D1 (p51) production cell line 10 were obtained from the Wistar Institute and analyzed for growth and production of neurons.

The four best growing clones were evaluated for production and yield of neurons which were further tested for the presence of TH by Western analysis. Only the neurons from the NT2/B9 and D1(P30) clones expressed TH at reproducibly detectable levels on Western blots and also were determined using the HPLC assay to produce HVA at levels similar to the hNT- 15 Neurons.

**5.B. The Effect of Maturation of hNT-Neurons on TH expression**

A study examined the effects of in vitro maturation of hNT-Neurons on TH levels. After the hNT-Neurons have developed during the RetA induction, cultures are routinely replated and 20 treated with mitotic inhibitors in the process of purifying the neurons (see Example 1). The hNT-Neurons were maintained in culture and allowed to mature either as a replate-I or as an enriched replate-II culture. To optimize the effects of neuron expression of TH, neurons were purified after culturing under different replate conditions and the levels of TH compared in the Western Blot assay. Samples were prepared from purified hNT-Neurons that had been treated 25 with inhibitors for 7 days as replate-I or replate-II cultures, and then maintained in growth media for a total of 1, 2, or 3 weeks of maturation. Extracts of the purified neurons were analyzed by Western Blot. The level of TH expression decreased dramatically with maturation in culture, and it was no longer detectable after 2 weeks in replate-II or after 3 weeks in replate-I. The levels of 30 TH found in the replate-I neurons not only were significantly higher, but also were expressed for a longer period. Control Western Blots were developed for each assay using an anti-Tau monoclonal to confirm that equivalent numbers of hNT-Neurons were loaded, and the samples were not degraded.

**5.C. Optimization of RetA induction for TH expression in hNT-Neurons**

TH expression paralleled the early development of hNT-Neurons and was evident by 3 to 4 weeks of RetA induction. Since the preliminary results showed that the TH expression level was greatly reduced upon maturation of hNT-Neurons for 2 to 3 weeks, a strategy was designed to determine if more TH was produced by less mature neurons. Possibly the hNT-Neurons that were produced after 5-6 weeks of RetA induction, which was optimal for the yield of cholinergic neurons, have been committed to down-regulate TH. To optimize production of hNT-Neurons for expression of TH and dopaminergic properties, a time course of RetA induction was performed and the TH levels in purified replate-I neurons from different RetA inductions were analyzed.

The NT2 precursor cells were induced with RetA to differentiate into hNT-Neurons. After RetA induction for 4, 5, or 6 weeks, the cultures were replated and treated with mitotic inhibitors for 7 days. The cells were refed with growth media and the neurons harvested either after 1 day (1 week replate-I) or after an additional 7 days (2 weeks replate-I). The extracts were prepared for denaturing SDS-PAGE and samples containing  $10^6$  cells/lane were transferred to Western blots for analysis.

The dramatic effect of RetA induction times on TH expression levels in the hNT-Neurons is shown in Figure 3. The expression of TH was found to be the highest in the replate-I neurons that were purified from the 4 week RetA Induction (Lane 2). The TH levels were seen to decrease significantly in purified neurons from 5 and 6 week RetA inductions (compare Lane 2 to Lanes 3 and 4). The loss of TH expression becomes even more evident in the 2 week matured hNT-Neuron samples from 4 week RetA (Lane 5) compared to week 5 and 6 (Lanes 6 and 7). These results demonstrate that there is an optimal RetA induction period of 3-5 weeks, perhaps peaking at 4 weeks, and also confirm that maturation in vitro reduces TH expression, even in the high expressing immature neurons (4 week RetA).

25

#### **Example 6. TH Expression of hNT Neurons treated with Lithium Chloride**

A small-scale dose range study analyzed whether therapeutic (0.5 - 1.0 mM) and clinically toxic (2.0 - 6.0 mM) concentrations of LiCl enhanced TH expression in cells treated for 6 weeks with retinoic acid and then LiCl. The control as well as lithium-treated cultures (0.5, 1.0 and 3.0 mM) was scored for TH immunoreactivity, which was expressed as the number of TH-positive (TH+) cells/well. For TH immunohistochemistry the cells were rinsed in 0.1 M phosphate buffered saline (PBS), fixed in 4% paraformaldehyde and then again rinsed in PBS. Before the primary antibody was applied, the cells were incubated in blocking serum (10% normal horse serum, in 0.02% Triton X-100 in 0.1 M PBS, pH 7.4) for one hour and then

incubated overnight in monoclonal antibody against TH (1:4000, INCSTAR, Stillwater, MN). The next day, secondary antibody, biotinylated horse antimouse (1:300, Vector, Burlingame, CA) was applied for one hour. The antibody complex was developed using avidin-biotin complex (ABC-Elite kit; Vector) and the developed product was visualized by using DAB (Pierce, Rockford, IL). To determine the percentage of cells labeled by a particular antibody in representative experiments, the numbers of labeled and unlabeled cells were assessed in a blind-coded manner using a 20X objective and a photographic frame. The frame was placed in 16 predetermined sites per well. This test was performed in duplicate.

Results are summarized in Figure 4. Of controls, TH+ neurons represented only 1% of the entire population of hNT cells. After treatment with 0.5 and 2.0 mM LiCl, almost 5% cells were TH+. The TH+ cells constituted nearly 7% of hNT cells treated with 1.0 mM of lithium. At higher concentrations (3.0 and 6.0 mM lithium), TH+ cells decreased to fewer than 2%. The treated cells are shown in Figures 5A through 5D. Arrows in Figure 5a point to individual TH+ neurons. In Figure 5b some cells reveal extremely long TH+ processes (arrowheads). Figures 15 5c and 5d show cultures of hNT cells treated with 3.0 mM of lithium. Cultures treated with 3.0mM of lithium grew equally well as the cultures treated with 1.0 mM lithium. Figure 5c shows several clustered hNT cells which were TH+ (arrows). Figure 5d shows intensely stained TH+ cells with well developed processes (arrow) which are typical of mature neurons. In summary, the results show that 0.5, 1.0 and 2.0 mM concentrations significantly enhanced the expression of TH in hNT neurons.

Next, purified hNT-neurons were tested after 4-week induction with RetA after mitotic inhibitor treatment. "Fresh Cells" had undergone Replate II treatment (see above). Prior to freezing, other cells underwent Replate I treatment. Control and lithium-treated cells (fresh or frozen) were cultured for five days and scored for TH immunoreactivity (see above method). The TH-immunostained slides were counterstained with propidium iodide to identify dead cells. Total numbers of TH+ and propidium iodide-positive (PI+) neurons were counted in control and lithium-treated cultures from standardized fields at 20X magnification, as described above.

Table 1. TH Expression in cultured (4wk fresh Replate II) hNT neurons

LiCl Dose	TH+ cells	Unlabeled Cells	Total Cells
Control	256 (56.1%)	209	465
1.0 mM	318 (85.3%)	53	371
3.0 mM	452 (76.8%)	138	590

Table 2. TH Expression in cultured (4wk thawed) hNT neurons

LiCl Dose	TH+ cells	Unlabeled Cells	Total Cells
Control	649 (70.4%)	272	921
1.0 mM	846 (79.1%)	224	1070
3.0 mM	623 (70.4%)	262	885

5

These results indicate that even 4-week RetA induced cultures of hNT neurons have significantly higher number TH+ cells (56-70%) than the usual six weeks (1%). These results are consistent with the Western analysis that also shows higher TH levels in 4-week compared to 6-week RetA neurons. In addition, Western analysis indicates that the levels of TH in fresh Replate II cultures are lower than that found in the frozen neurons. (Table 2). Exposure to 1.0 mM LiCl significantly increased the number of TH+ cells to 80-85%. When no primary antibody was added to the negative control cultures (primary delete), the control cultures were immunonegative.

Photomicrographs (Figs. 6a, 6b and 6c) show representative control 5 days in cultures of hNT neurons previously treated for four weeks with RetA. Fig. 6a is a control culture of hNT neurons immunostained with antibodies to TH which had a significantly higher number of TH+ cells in comparison to controls treated for six weeks with RetA (See Fig. 4). Fig. 6c is a control culture of hNT neurons immunostained with antibodies to bcl-2 to demonstrate the co-localization of an anti-apoptotic gene with TH expression.

Incubation of hNT cells with LiCl increased the size of the TH neurons as well as the length and number of processes. In addition, there was a 5-fold increase in TH expression, with the greatest effect occurring at 1 mM concentration. At this dose, almost 7% of the hNT neurons population were TH+ (Zigova T, et al., LiCl induces the Expression of TH in hNT Neurons, Poster to be presented at Society for Neuroscience 1998 Annual Meeting in Los Angeles, CA, Nov. 7-12, 1998).

#### **Example 7. Viability of hNT Neurons treated with Lithium Chloride**

The effect of LiCl on hNT viability was assessed using the Trypan blue exclusion technique (blue dead cells) and a double-staining procedure using fluorescein diacetate (FDA) and propidium iodide (PI), according to Jones and Senft (J Histochem Cytochem 33:77-80, 1985). The hNT cells were seeded in 10 $\mu$ g/mL poly-L-lysine-coated 8-well chamber slides at a concentration of 100,000 hNT neurons/cm<sup>2</sup> in a DMEM medium supplemented with 10% FBS and 50 g/mL gentamicin (Sigma). Plated cells were maintained in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 90% air at 37°C). After 24 hours, the media was changed to DMEM:F12 containing 0.1% ITS (Sigma), gentamicin and LiCl (Sigma) at 0, 1.0 and 3.0 mM concentrations. The cells

were incubated for an additional four days. Then, trypan blue was added to the medium. In three random fields from control and both lithium-treated cultures, the numbers of viable and dead cells were counted.

5 Viability of surviving cells in 1.0 mM and 3.0 mM LiCl was 71.55 and 70.15 (2.65% and 2.17%, respectively), compared to untreated hNT control cell viability of 74.22 (1.56%). Thus, the survival of hNT cells cultured for five days with LiCl was not significantly changed.

#### **Example 8. Lithium Induction of bcl-2 Expression**

Because the proto-oncogene bcl-2 has been shown to protect a variety of cell types from programmed cell death, it is often considered an inhibitor of apoptosis (Sentman et al. 1991).  
10 Lithium-treated cells for the involvement of bcl-2 which could help protect hNT neurons. First, the immunocytochemical expression of bcl-2 protein in hNT cells cultured for 5 days with 0.5 and 3.0 mM LiCl was evaluated. Immunostaining was performed as described for TH (above), except that the monoclonal antibody to bcl-2 (Ab-1, 1:50, Calbiochem, Oncogene Research  
15 Products, Cambridge MA) was used. Figure 7 summarizes the effects of different doses of LiCl on bcl-2 immunostaining. These values were expressed as the number of bcl-2+ cells/well and clearly indicated significantly enhanced expression of anti-apoptotic agent in LiCl-treated hNT cells.

Secondly, the number of bcl-2+ cells versus total number of cells in experimental (0.5 and 3.0 mM) and control groups were compared. Among control cells, 19.14% (288/1504) were bcl-2+; among 0.5 mM treated cells, 31.62% (278/879) were bcl-2+; and among 3.0 mM treated cells, 29.50% (562/1903) were bcl-2+. These results indicate that lithium enhances bcl-2 expression in hNT cells and thus may act as a neuroprotective agent.  
20

#### **25 Example 9. Effects of Lithium Chloride on hNT Cell Line**

In the present study, lithium chloride in several doses was tested for its ability to induce the expression of tyrosine hydroxylase (TH) in neurons derived from a human teratocarcinoma cell line (hNT) after 5 and 10 days in vitro (DIV). The data suggests that hNT cells are indeed responsive to lithium exposure and may serve as a continual source of TH-expressing neurons in new therapeutic approaches to degenerative brain disease.  
30

#### **Example 9.A. Preparation of hNT Neurons**

The hNT neurons (Layton Bioscience, Inc., Atherton, CA), previously treated for 6 weeks with retinoic acid, were stored at -80°C prior to use. Cells were thawed rapidly at

37 C and transferred into a 15-cc tube containing DMEM (Gibco, BRL, Grand Island, NY) and 10% fetal bovine serum (FBS; Gibco, BRL). After centrifugation (1000 rpm/7 min), the cells were resuspended in 1 ml of the above fresh media and the cell number assessed with a trypan blue exclusion method. Cells were plated at a concentration of 100,000/cm<sup>2</sup> on eight-well poly-L-lysine (10 g/ml, Sigma, St. Louis, MO) coated chamber slides (Nunc, Naperville, IL) at 5 37°C in 5% CO<sub>2</sub> at 95% humidity. After 24 h the media was switched to DMEM:F12 (Gibco, BRL) containing 0.1% ITS (Sigma), Gentamicin (50 µg/ml, Sigma) and varying concentrations (0.5, 1.0, 1.5, 2.0, 3.0 or 6.0 mM) of lithium chloride (LiCl; Sigma). In the first culture series, maintaining medium was supplemented with LiCl in various concentrations and cultures were maintained for an additional four days. Sister cultures were maintained up to 10 days in vitro (DIV) with a switch to fresh media without lithium chloride on day five. In the second culture 10 series, NaCl (Sigma; 1.0 mM) or KCl (Sigma; 1.0 mM) was added to the medium, rather than LiCl, and cells were maintained in culture until day 5. Each series was performed in three independent cultures. For immunocytochemistry all cultures were rinsed in 0.1 M phosphate 15 buffered saline (PBS) and fixed with 4% paraformaldehyde (pH 7.4).

#### **Example 9.B. Immunocytochemistry**

Immunocytochemistry was performed using monoclonal primary antibody against growth-associated protein (GAP-43, 1: 8000; Chemicon, Temecula, CA), GAP-43 is a neuronal marker highly expressed during neuronal differentiation and neurite outgrowth (Skene JHP, Ann Rev Neurosci 12: 127-156, 1989; Strittmatter SM, Vartanian T, Fishman MC, J Neurobiol 23: 507-520, 1992). In our study it was used to demonstrate the morphological appearance of cultured hNT neurons. Monoclonal antibody against tyrosine hydroxylase (TH, 1: 4000; INCSTAR, Stillwater, MN) was used to determine the neurotransmitter phenotype of hNT cells 20 in culture. For both primary antibodies, biotinylated horse-anti-mouse (1:300; Vector, Burlingame, CA) secondary antibody was used. The antibody complex was developed using an 25 avidin-biotin kit (ABC-Elite kit; Vector) and the developed product visualized by using 3,3' – diaminobenzidine (DAB; Pierce, Rockford, IL).

#### **30 Example 9.C. Effect of Lithium on TH expression in hNT Neurons**

To investigate the effect of various doses of LiCl on tyrosine hydroxylase expression in 5 days in culture of hNT neurons we determined the number of unlabeled and TH-positive neurons in a blind coded manner using a 20X objective and a photographic frame (field = 0.3 mm<sup>2</sup>). The frame was placed in 16 predetermined sites per well. The counts of TH-positive.

neurons in lithium-treated cultures were compared with counts obtained from cultures treated with NaCl or KCl. The dose responsive tests were analyzed using a one-way analysis of variance (ANOVA) followed by post hoc comparisons using Bonferroni-Dunn analysis.

Following immunocytochemistry for tyrosine hydroxylase, 5 days in culture, TH-positive hNT neurons could be detected throughout the culture dish (Fig. 10). In control cultures, most TH-positive hNT neurons revealed oval or spindle-shaped soma with short and smooth neurites preferentially emerging from basal and apical parts of the soma. In some instances, TH-positive neurons did not display processes at all (Fig. 10A). In lithium-treated cultures, the TH-positive cell bodies were larger and their neurites substantially longer with numerous varicosities (Fig. 10D, 10E).

To determine whether expressions of the tyrosine hydroxylase in cultured hNT cells could be enhanced by using therapeutic (0.5 – 1.0 mM) and clinically toxic (2.0 – 6.0 mM) concentrations of lithium chloride, the control as well as lithium treated cultures were scored for TH immunoreactivity. The counts were expressed as the percentage of TH-positive cells in relation to the total number of hNT cells per field. In 6-week controls, TH-positive neurons represent only 1% of the entire population of hNT cells. Two therapeutic concentrations of lithium, 0.5 and 1.0 mM, significantly increased ( $p < 0.01$ ) the percentage of TH-positive neurons per field (5 and 7%, respectively). TH expression was slightly lower in hNT cells after treatment with 2.0 mM concentration of lithium. In the last two concentrations (3.0 and 6.0 mM) the TH-positive neurons represented about 2% of the entire population of hNT cells (Fig. 11).

Photomicrographs of representative control and lithium-treated hNT neurons cultured for 5 days and immunostained for tyrosine hydroxylase are shown in Fig. 11A, control culture of hNT neurons reveals few TH-positive cells (arrow). The bar represents 50  $\mu$ m. (Fig. 11B and 11C) show hNT cells cultured with 1.0mM (Fig. 11B) and 3.0 mM (Fig. 11C) lithium chloride; arrows point to individual TH-positive neurons. In lithium-exposed cultures more TH-positive cells with longer TH-immunoreactive processes (arrowheads) were present. Figures 11D and 11E show the representative morphology of TH-positive hNT cells treated with 1.0mM (Fig. 11D) and 3.0 mM (Fig. 11E) of lithium chloride. Even though only cultures supplemented with 1.0mM dose of lithium chloride increased the overall yield of TH-positive hNT neurons, both the 1.0 mM and 3.0 mM doses promoted morphological development. Frequently, intensely TH-positive cells with well-developed processes (arrowhead), typical of mature neurons were found.

In order to determine whether the induction of tyrosine hydroxylase expression would be

sustained if lithium chloride administration was terminated, the number of TH-positive neurons on day 10 was assessed. The control cultures and cultures exposed to 1.0 mM lithium chloride for 5 days, received new media lacking LiCl and were maintained until day 10. Control cultures revealed a relatively low percentage of TH-positive cells (less than 2%), while cultures exposed to 5 1.0 mM lithium chloride displayed the values comparable to values obtained on day 5 (ranging between 5-7%). This indicated that hNT cells were able to maintain the expression of tyrosine hydroxylase even though the inducing agent LiCl was absent from the media after day 5.

In a parallel series of experiments, the effect of NaCl or KCl (1.0 mM concentrations) 10 was compared to that of LiCl (1.0 mM) on TH expression in 5 days in cultures of hNT neurons. The numbers of TH-positive hNT cells in cultures treated with either NaCl or KCl were not significantly different from values obtained in control (not supplemented with lithium). From these results it could be concluded that lithium and not the chloride (salt) was responsible for the induction of TH expression in hNT neurons.

The findings of this study demonstrated that lithium could induce the expression of TH 15 in cultured hNT neurons. First, it was shown that the induction of the TH expression in hNT cells was dose-dependent. Second, the effect of lithium treatment in the optimal dose (1.0 mM) was sustained even after the treatment with the inducing agent was discontinued.

Previous reports have demonstrated the induction of tyrosine hydroxylase expression in 20 cultures of primary brain neurons (Du X, Stull ND, Iacovitti A, Brain Res 680: 229-233, 1995); however, the importance of finding alternative dopameric sources prompted the idea to induce the expression of TH in other non-TH expressing neurons. In our lab, a co-culturing hNT cells with Sertoli cells also clearly induced TH production (Othberg AI, Willing AE, Cameron DF, Anton A, Saporta S, Freeman TB, Sanberg PR, Cell Transplant 7: 157-164, 1998). 25 Together with the above mentioned findings our present results showing the induction of TH expression after lithium treatment support the idea that this cell line, previously low in intrinsic TH expression, can be converted into significantly high levels of TH expressing cells.

In the present study, lithium, an effective psychotherapeutic agent induced the expression 30 of TH in hNT cells. Not wishing to be bound by a particular theory, the increased TH expression can be due to increased TH synthesis and/or increased Th activity, both of which are mediated by signal transduction pathways including protein kinase C (Zigmond RE, Schwarzschild MA, Rittenhouse AR, Ann Rev Neurosci 12:415-461, 1989).

#### **Example 9.D. Effect of Lithium on Size and Neurite Outgrowth of hNT Neurons**

The same series of slides immunostained for TH were employed to measure the soma size ( $\mu\text{m}^2$ ) and neurite outgrowth ( $\mu\text{m}$ ) of appropriately 50 TH-positive cells per representative culture experiment using a computerized image analysis program (Image-Pro Plus, Media Cybernetics, Inc., Silver Springs, MD) at 20X objective. The results in the morphological assessment study are reported as mean  $\pm$  SEM and were analyzed using Student's t-test. The size of the TH-positive cell bodies increased significantly ( $p < 0.01$ ) after application of 1.0 and 3.0 mM dose of lithium chloride, ranging from  $33.8 \mu\text{m}^2$  to  $103.3 \mu\text{m}^2$  (mean =  $64.1 \pm 2.5 \mu\text{m}^2$ ) in the control group and from  $53.09$  to  $183.3 \mu\text{m}^2$  (mean =  $103.2 \pm 2.7 \mu\text{m}^2$ ) and from  $61.4$  to  $165.8 \mu\text{m}^2$  (mean =  $104.8 \pm 3.2 \mu\text{m}^2$ ) in 1.0 and 3.0 mM lithium-treated groups, respectively  
5 (Fig. 12A). Soma sizes in 5 days in culture NaCl or KCl-treated cultures were not significantly different (Fig. 12A) from control.  
10

The second parameter characterizing the effect of lithium on the development of TH-positive hNT cells was neurite outgrowth. The length of processes in controls ranged between  $10.4 \mu\text{m}$  (mean =  $25.02 \pm 2.9 \mu\text{m}$ ), while in both groups exposed to lithium significantly ( $p <$   
15  $0.01$ ) longer processes were found (Fig. 10E, 10D). In cultures treated with 1.0 mM dose of lithium chloride the lengths ranged between  $12.2 \mu\text{m}$  and  $87.3 \mu\text{m}$  (mean =  $43.4 \pm 2.8 \mu\text{m}$ ) and in the group treated with 3.0 mM concentration the lengths varied between  $20.3 \mu\text{m}$  and  $128.1 \mu\text{m}$  (mean =  $52.9 \pm 3.8 \mu\text{m}$ ). Neurite outgrowth in NaCl and KCl-treated 5 days in cultures did not significantly differ from control values (Fig. 12B). These results clearly demonstrated that  
20 morphological development was significantly enhanced in TH-positive hNT cells treated with both lithium concentrations.

Soma size and neurite outgrowth were also measured in hNT cells maintained in culture for 10 days and treated for 5 days with the most effective dose of lithium (1.0 mM). The mean soma size of TH-positive hNT cells was significantly larger ( $102.8 \pm 2.5 \mu\text{m}^2$ ) ( $p < 0.01$ ) than in  
25 5 days in cultures, but did not differ significantly from the mean value of the lithium-treated group ( $103.2 \pm 2.7 \mu\text{m}^2$ ). The mean length of neurite processes in controls was  $24.8 \pm 2.4 \mu\text{m}$  which was not different from younger (5DIV) control cultures but significantly different from 10 day lithium-treated group ( $55.5 \pm 5.1 \mu\text{m}$ ). In addition, as a result of lithium treatment, numerous TH-positive cells revealed multiple branching processes with varicosities.  
30 Collectively, these results suggested that TH-converted hNT cells responded to lithium treatment by enhancing morphological maturation at both time points studied.

Morphometric analysis revealed that TH-positive cells in cultures exposed to lithium resulted in significantly enlarged soma size and longer neurites, as well as a higher degree of neuronal complexity. Taken together, our results suggested that the most effective

concentration of lithium (1.0mM) was adequate to induce TH expression and morphological development of cultured hNT neurons.

**Example 9.E. Effect of Lithium on Viability of hNT Neurons**

5        The effect of lithium chloride on the viability of hNT neurons was evaluated in living, trypan blue stained cultures. Our preliminary results (Zigova et al. Abst. Neuroscience 24: \_\_\_\_ 1998) are in agreement with reports of other authors (Terao et al., Biol. Psychiatry 31: 1038-49, 1992) and indicated that 1.0 and 3.0 mM concentrations of lithium chloride had little effect on cell morphology. In addition, the lower dose was within the therapeutic range (0.5 – 1.0 mM),  
10 while the higher dose was within the range of clinically toxic concentrations (2 – 4 mM). To assess the effect of these two doses of lithium chloride on hNT neurons, the trypan blue exclusion in living cultures and GAP-43 immunohistochemistry or paraformaldehyde-fixed cultures were employed.

For the trypan blue exclusion, the numbers of dead (blue) and living (unstained) cells  
15 were counted and their percentage calculated in 10-15 randomly chosen microscopic fields in 3 separate wells per condition at 10x magnification. The data from control and lithium-treated cultures were compared using the unpaired *t* test. The number of surviving hNT neurons was estimated 5 and 10 days in culture control and lithium-treated cultures to detect any possible deleterious effect of lithium chloride. The cells cultured for 5 to 10 days without any supplement  
20 were compared to those treated either with 1.0mM or 3.0mM dose of lithium chloride. In 5 day control cultures the percentage of viable cells was about 74% and did not significantly differ from cultures treated either with 1.0 (71%) or 3.0mM lithium chloride (70%). (Fig. 9A) Phase contrast low-magnification photomicrograph showing the distribution of hNT neurons in control/untreated cultures. Note that these cells have a tendency to aggregate into clumps of  
25 variable sizes. At 5 days the majority of hNT cells have long processes emanating from their soma. This bar represents 100  $\mu$ m.. The overall cell viability ranged between 79-84% in cultures maintained until day 10, with media replacement of day 5.

The effect of lithium on survival of hNT neurons was also evaluated from cultures fixed and immunostained for neuronal marker, growth-associated protein GAP-43. The actual counts  
30 were obtained by placing the photographic frame of the microscope over five randomly chosen fields (field size = 0.2 mm<sup>2</sup>) in each well at 200 x magnification. The mean number of GAP-43-positive cells per field was calculated from 4 wells per condition. The immunostaining was selected as further confirmation of hNT's neuronal phenotype and to facilitate the neuronal counts. Morphologically, GAP-43 positive hNT neurons usually exhibited round or oval

perikarya and neurites including growth cones (Fig. 9). In Fig. 9B, higher magnification shows that virtually all cultured hNT cells are immunoreactive for GAP43, and thus neuronal phenotype. GAP-43-positive hNT cells usually have oval-shaped cell bodies with one or two processes, some of which demonstrate growth cones. The bar represents 50  $\mu$ m.

5 Independent of survival time or lithium dose used, hNT neurons were aggregated into tightly or loosely packed clusters frequently interconnected with each other. (Fig. 9C, 9D) Low-magnification phase contrast photomicrographs of hNT neurons treated with 1.0 mM (Fig. 9C) and 3.0 (Fig. 9D) concentration of lithium chloride. In both experimental groups the aggregation pattern and morphological appearance of cultured hNT neurons was similar to 10 cultures unexposed to lithium treatment. (Fig. 9E) Morphological appearances of GAP-43-labeled hNT neurons treated with 3.0mM lithium chloride demonstrating GAP-43-positive cell bodies with prominent growth cones (arrowheads) similar to those observed in cultures not supplemented with lithium chloride. The bar represents 25  $\mu$ m.

In addition, GAP-43 immunostaining facilitated the neuronal counts on these cultures, 15 whose aggregates hampered the cell counting if unstained. Typically, in the 5 days in hNT control cultures the number of viable neurons varied between 110-140/ field that was similar to cultures receiving 1.0 and 3.0 mM lithium chloride. In control 10 day-old-cultures and cultures treated with 1.0mM lithium chloride, the individual counts per field ranged from 110-150 and in the group treated with 3.0mM lithium chloride these varied between 100-140 / field. These 20 counts were not statistically significantly different from the control values. When the mean number of neurons / field was used to calculate the total number of neurons per well in control cultures, it was shown, that there were about 50,000 – 54,000 cells / well at both 5 and 10 days. As the initial cell plating in all groups was 89,000 cells / well it suggests that there was an approximately 30 – 40% loss of cells caused by their detachment from the surface of the dish. 25 Taken together, these findings indicated that the presence of 1.0 or 3.0 mM of lithium chloride had no deleterious effect on the survival of hNT neurons in vitro.

The second important finding of this study was that the most effective TH inducing dose of lithium (1.0 mM) was not detrimental to cultured hNT neurons. This dose is within the range of therapeutic concentrations (0.5 – 1.0mM) (Johnson, Depression & Mania, Oxford IRL, 1987), 30 and in addition to being employed in treatment of mood disorders, lithium is a neuroprotective agent against a variety of neurological deficits. A neuroprotective effect of chronic lithium administration on focal cerebral eschemia was recently shown by Nonaka and Chuang (Neuroreport 9:2081 – 2084,1998). The authors assumed that chronic lithium-induced neuroprotective benefit is probably due to its ability to attenuate excessive calcium influx

mediated by NMDA receptors. They also reported that chronic lithium treatment (at therapeutically relevant concentrations of this drug – 1.3 mM robustly protected cultured CNS neurons against excitotoxicity mediated by NMDA receptors (Nonaka et al., PNAS 95:2642-2647, 1998). An anti-apoptotic effect of lithium on cultured cerebellar granule cells has also been reported after application of anticonvulsant (Nonaka et al., 1998) and KCL deprivation (D'Mello et al. Exp Cell Res 211:332-338, 1994). Further studies demonstrating either enhanced expression of neuroprotective genes or decreased expression of pro-apoptotic genes in lithium-treated hNT cells are necessary to confirm the possible neuroprotective effect of lithium.

In summary, the present results indicate that lithium does indeed increase the TH activity  
10 of hNT cells.

#### **Example 10. Process for LiCl Induction of DA Neurons**

NT-2/D1 Precursor cells were induced with 10  $\mu$ M RetA for four weeks. The cultures were replated and treated with mitotic inhibitors for seven days. Purified DA-Neurons were  
15 selectively harvested. Approximately  $2 \times 10^6$  neurons were plated in flasks that contained one of the following feeder cultures of mouse 3T3 cells, TM-4 cells (a Sertoli cell line), NT-2 Accessory cells, primary human astrocytes or primary rat glial cells. These co-cultures were maintained for seven days, after which the mature neurons were differentially harvested for the feeder cultures. Extracts were prepared from about  $0.5 \times 10^6$  Cells, which were reduced,  
20 denatured and analyzed for TH using a Western blot technique.

The levels of expressed TH are shown in Figure 13 (DA-Neuron Co-Culture). Lane 1 is a control of co-cultured 500 pg of TH, lane 2 shows TH from fresh DA-Neurons bulk harvested. Also shown are TH from DA cells 1-week with polylysine/Laminin (lane 3), TM-4 cells (lane 4) and rat glial cells (lane 5).

25 Also shown in figure 13 is that TH levels decreased dramatically from that found in the fresh DA-neurons (lane 2) in cultures plated on polylysine alone, or on rat glial cells (lane 5). Similar results were found for most of cells with the exception of the TM-4 cells (lane 4). The level of TH expressed in DA-Neurons co-cultured for 1 week with the TM-4 cells was comparable to fresh neurons (lane 2). The stabilization of TH levels in the TM-4 co-cultured  
30 neurons appears to be aided by cell-cell interactions, since TM-4-conditioned media had no effect.

#### **Example 11. Other TH-Enhancing Treatments**

In addition to the above experiments to enhance and stabilize the high level of TH that is

expressed in the hNT neurons (i.e., DA/hNT-neurons), additional experiments are undertaken, including different feeder cells and other cytokines and neurotrophic factors. A number of different feeder cell layers are compared for the maintenance of purified neurons and the enhancement of TH expression during maturation of the neurons. The effects of treating the  
5 DA/hNT neurons with cytokines (e.g., FGF, TGF and LIF) and neurotrophic factors (e.g., BDNF, GDNF, NT4/5) on the development of their dopaminergic properties in addition to TH levels are also investigated. The DA/hNT-neurons from the best plating and enhancement protocols are then analyzed for production of DA using the HVA assay. The optimal process/treatment regimen is then scaled up for production of DA/hNT neurons for testing in  
10 animal studies.

#### **EXAMPLE 12. Production of LiCl Induced DA-Neurons**

The enhanced expression of TH in the 4-week DA-Neurons was found to be optimal in small scale cultures after replating the neurons in serum-free media containing 1 mM LiCl for 5  
15 to 7 days. The neurons analyzed in these initial studies were purified and frozen prior to plating on polylysine for testing, conditions where TH levels decrease without treatment. It would be ideal to incorporate the LiCl induction into the DA-Neuron process to obtain 4-week RetA induced DA-Neurons that are also treated with LiCl. To determine the conditions for production of LiCl induced DA-Neurons, a series of LiCl treatments were designated to evaluate conditions  
20 for optimal TH expression.

The purpose of this experiment was to determine on 4-week RetA cultures the effect of LiCl treatment during the Replate I mitotic inhibition step of the process, which occurs just before harvest of the DA-Neurons. Replate cultures were treated with 1 mM LiCl in the presence of inhibitors (FudR & AraC) for the standard 7 days of replate or during the last three  
25 of the 7 days (prior to harvest). The 3-day LiCl treatment was also evaluated without mitotic inhibitors and in serum-free media (+ITS). After treatment the neurons were selectively harvested, processed and TH levels analyzed using Western Blots. The 7-day-LiCl Neurons contain about 50% higher levels of TH than the 7-day inhibitor only control (compare Fig. 14 Lanes 2 and 3). Surprisingly, the 3-day-LiCl neurons also expressed levels of TH comparable to  
30 the control, with the uninhibited sample (lane 5) expressing somewhat more TH. The neurons harvested from serum-free media expressed significantly less TH (Fig. 14 D/F+ITS, lanes 6 and 7). Serum-free neurons may not have developed as well and were contaminated with accessory cells.

Figure 14 shows the results for 500 pg TH (lane 1); control DA neurons in DF-5/Inh, .7

days (lane 2); DA neurons in DF-5/Inh/ 1mM LiCl, 7 days (lane 3); DA neurons in DF-5/Inh/ 1mM LiCl, 3 days (lane 4); DA neurons in DF-5/1mM LiCl, 3 days (lane 5); DA neurons in DF-5/ 1mM LiCl, 3 days (lane 6); and DA neurons in DF/ 1% ITS/ 1mM LiCl, 3 days (lane 7). DA neurons treated with LiCl (lanes 3, 4 and 5) seemed to express more TH than did the DA  
5 neurons maintained in DF-5/Inh alone (lane 2). DA neurons with 7 days of LiCl (lane 3) and with 3 days of LiCl (lane 4) had similar levels of TH. The weaker signals in lanes 6 and 7 of Fig. 14 may be due to higher contamination with accessory cells. During harvest of these flasks, the accessory cell layer came off more rapidly than it did from other flasks.

10 **Example 13.**

As disclosed above in example 2, different cells can be adapted for increased TH and dopaminergic production. The initial step is analyzing the cells' TH activity; examples of additional cell types are listed below. Subsequently, additional experiments as discussed above are undertaken to change plating, maturation and co-culturing techniques and thus optimize each  
15 cell type's TH activity.

U.S. Patent No. 5,639,618 discloses a stable line of lineage-specific neuronal stem cells. The stem cells are constructed from blastocyst-derived ES cells transfected with a reporter construct under the control of the Otx regulatory region, Otx being an early marker for neurogenesis. The reporter construct is used to segregate the neuronal stem cells by FACS  
20 isolation or other method. The segregated cells are then plated and permitted to terminally differentiate.

U.S. Patent No. 5,618,531 discloses a method for increasing the viability of cells, which are administered to the brain or spinal cord. The method is accomplished by attaching the cells to a support matrix and implanting the support matrix into the brain.

25 U.S. Patent No. 5,135,956 discloses using long-chain (23 to 29 carbons) fatty alcohols and prodrugs esters to cause extension of neurites in vivo and facilitate healing of traumatic injury to both the central and peripheral nervous systems by facilitating reconnection and reestablishment of function, decreasing ischemia and neuronal death, and reducing Wallerian degeneration after injury.

30

**Example 14. TH-Enhanced hNT-Neurons in PD rat model**

The most widely used model to evaluate the potential of neural transplantation to relieve the symptoms of PD is the rotational model of nigrostriatal function in rats (Koutouzis TK, Emerich DF, Borlongan CV, Freman TB, Cahill DW, Sanberg PR. Critical Reviews in

Neurobiology 8: 125-162, 1994). In this model, the toxin 6-OHDA is stereotactically injected into the substantia nigra to destroy dopaminergic neurons and to reduce TH activity on one side, creating a unilateral DA receptor supersensitivity. Lesioned animals develop stereotypical rotational behavior contralateral to the lesioned side in response to apomorphine, and ipsilateral to amphetamine that stimulates DA release. Reduced numbers of rotations per unit of time reflect greater treatment efficacy. The goal of the present pilot study was to demonstrate a dose-response effect of transplants of hNT-Neurons on TH expression and rotational behavior.

hNT-Neurons were co-cultured with Sertoli cells and showed upregulation of TH expression compared to mono-cultures of hNT-Neurons. To confirm this effect *in vivo*, a study 10 investigated the effects of transplanting hNT-Neurons, alone or in combination with Sertoli cells, into the striatum of 6-OHDA-lesioned hemiparkinsonian rats. Briefly, 15 Sprague-Dawley rats underwent 6-OHDA lesioning and later were assessed for apomorphine-induced rotations. Transplantation was done by stereotaxic injection into the striatum. Transplants consisted of 15 hNT-Neurons alone, hNT-Neurons and Sertoli cells together, or hNT-Neurons co-cultured with Sertoli cells for 72 hours prior to transplantation. Doses of approximately  $10^6$  viable cells of either cell type were used. Functional recovery was measured by fewer post-transplantation turns in response to apomorphine.

Preliminary results showed significant functional recovery in all groups. The number of rotations decreased significantly with time ( $F=14.3$ ;  $p<0.001$ ). At 2 weeks post-transplantation, 20 all animals showed significant improvement in apomorphine-induced rotations ( $F=22.3$ ;  $p<0.001$ ) which continued to improve at 4 and 12 weeks. The similar performance levels between the two groups may be due to the small sample sizes. Importantly, this is also the first time that behavioral recovery occurred with hNT-Neurons in the absence of immunosuppression. Perhaps hNT-Neurons themselves possess immunosuppressive properties, 25 or perhaps they are not recognized by the host as "non-self," which might be due to their injection into the immunologically privileged brain or to the cells' derivation from a cancer cell line. Histological analysis of immunocytochemically-detectable TH, OX-42, GFAP and MOC-1 to assess survival and immune response is currently ongoing. The final results will help determine the number of surviving hNT-Neurons, the host immune response to the transplanted 30 cells, and whether the Sertoli cells provided trophic or immune support to the hNT-Neurons to increase the TH signal.

An alternative transplant method is given in Ivar Mendez, Damaso Sadi and Murray Hong, J. Neurosci. 16(22):7216-27, 1996 (incorporated herein by reference).

**Example 15. MPTP non-human model of PD**

A non-human higher mammal study (e.g., in primates) bridges the gap between rodent and human. This study demonstrates efficacy, long-term effects, scale-up to doses close to humans, general and specific safety, and valid neurosurgical procedures in preparation for clinical trials for the indication of PD. Non-human primates may be unilaterally lesioned in the caudate and putamen with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to produce hemiparkinsonian-like symptoms that provide a good model for evaluation of interventions in PD. Following the lesion, animals spontaneously circle in the direction of the lesion, but in response to apomorphine, they reverse and circle contralateral to the side of the lesion.

Associated motor and behavioral symptoms include generalized slowing, rigidity and tremors characteristic of PD. After the lesions stabilize, optimized DA/hNT neurons are administered to at least one part of the lesioned area. Possible administration protocols call for delivery of the neurons in 1-20 tracks, preferably 1-10 tracks, more preferably 1-6 tracks and most preferably about 4 tracks. It is understood that one or more doses of cells is administered in each track, once as the needle is initially placed and optionally again or repeatedly as the needle is partially withdrawn. The amounts of cells to be delivered in each track varies from about  $5 \times 10^4$  to  $5 \times 10^6$  cells per dose, preferably  $1 \times 10^5$  to  $2 \times 10^6$  cells per dose, most preferably about  $5 \times 10^5$  to  $1.5 \times 10^6$  per dose, and most preferably  $10^6$  per dose; however, these dose ranges are to be modified based on the identification of the optimal dose in rodents and the appropriate scale-up factor for higher mammals. The cells or appropriate control(s) are administered to caudate and putamen of MPTP-lesioned animals. Animals undergo repeated behavioral testing at suitable intervals before being sacrificed for histological analyses. Histologic examination will be done for tumor formation, brain infection, and graft rejection.

While not wishing to be bound by any theory, one possible explanation for the effectiveness of hNT-Neurons in multiple brain disorders is that the morphology and differentiation of the TH-induced, transplanted hNT-Neurons may be determined by the microenvironments of the CNS region of the host into which they are introduced. The ability of hNT-Neurons to migrate following transplantation was investigated (Zigova and Luskin, in preparation) by transplanting fluorescent-labeled neurons into the subventricular zone (SVZa) of newborn rat pups. The cells migrated tangentially along a stereotypical and extended pathway to the olfactory bulb, and then turn radially into one of the overlying neuronal cellular layers.

Fluorescence microscopy and immunohistochemistry revealed aggregates of labeled cells at the site of implantation in the SVZa, as well as along the migratory pathway leading to the olfactory bulb, and in the subependymal zone in the middle of the olfactory bulb. None of the

labeled cells deviated from the pathway. Some of the transplanted labeled neurons exhibited a neuronal morphology with distinct processes and stained with the antibody TuJ1, a neuron-specific marker, suggesting that the environment of the SVZa and the pathway leading to the bulb provided cues guiding the differentiation of hNT-Neurons.

5       The foregoing description and examples are intended only to illustrate, not limit, the disclosed invention.

Claims

1. A method of producing dopaminergic neuronal cells suitable for transplantation in dopamine deficiencies, said transplantable neuronal cells being derived from progenitor cells,
  - 5 a. providing progenitor cells which lack at least one indicator of neuronal cell differentiation;
  - b. treating the progenitor cells with an inducing agent for a time period sufficient to optimize expression of tyrosine hydroxylase and to induce the presence of at least one indicator of neuronal cell differentiation to produce a plurality of dopaminergic, differentiated neuronal cells; and
  - 10 c. harvesting the dopaminergic, differentiated neuronal cells.
2. The method of claim 1, wherein the step of providing progenitor cells provides mammalian cells.  
15
3. The method of claim 1, wherein the step of providing progenitor cells provides NT2 neuron cells.
4. The method of claim 1, wherein the step of providing progenitor cells provides  
20 mammalian fetal cells.
5. The method of claim 1, wherein the step of providing progenitor cells provides mammalian stem cells.  
25
6. The method of claim 1, wherein step (b) is followed by an additional step of adding at least one lithium chloride salt.
7. The method of claim 1, wherein step (b) is followed by an additional step of adding Sertoli cells.  
30
8. The method of claim 1, wherein step (b) is followed by an additional step of adding astrocytes.
9. The method of claim 1 wherein step (b) is followed by an additional step of

adding glial cells, astrocytes, accessory cells or a combination thereof.

10. The method of claim 1 wherein the step of treating the progenitor cells comprises applying retinoic acid or retinoids thereto.

5

11. A dopaminergic neuronal cell suitable for transplantation into an individual having a dopaminergic deficiency, said cell comprising

a post-mitotic differentiated neuronal cell which expresses tyrosine hydroxylase and at least one other indicator of neuronal cell differentiation, said cell having undergone induction  
10 from an undifferentiated cell.

12. A human post-mitotic dopaminergic cell suitable for transplantation into a human having a dopaminergic deficiency, said cell comprising a differentiated neuronal cell which expresses tyrosine hydroxylase and at least one other indicator of neuronal cell differentiation,  
15 said cell having undergone induction from an undifferentiated human cell.

13. A human dopaminergic cell suitable for transplantation into a human having a dopaminergic deficiency, said cell comprising a differentiated human neuronal cell which expresses tyrosine hydroxylase and bcl-2, said cell being capable of synthesizing dopamine and  
20 having improved survival after transplantation.

14. A method of improving the survival of human neuronal cells for transplantation, said method comprising the steps of

- a. providing a culture of human cells;
- 25 b. adding a lithium salt to the human cell culture for a sufficient time to enhance expression of bcl-2;
- c. testing cells from the treated cell culture for the presence of bcl-2; and
- d. isolating the cells from the culture to produce an isolated cell preparation; and
- e. testing the isolated cell preparation for sterility before packaging the cells for  
30 transport.

15. A pharmaceutical dosage form of human cells suitable for transplantation comprising

isolated and purified human cells said cells having been treated with a lithium salt to

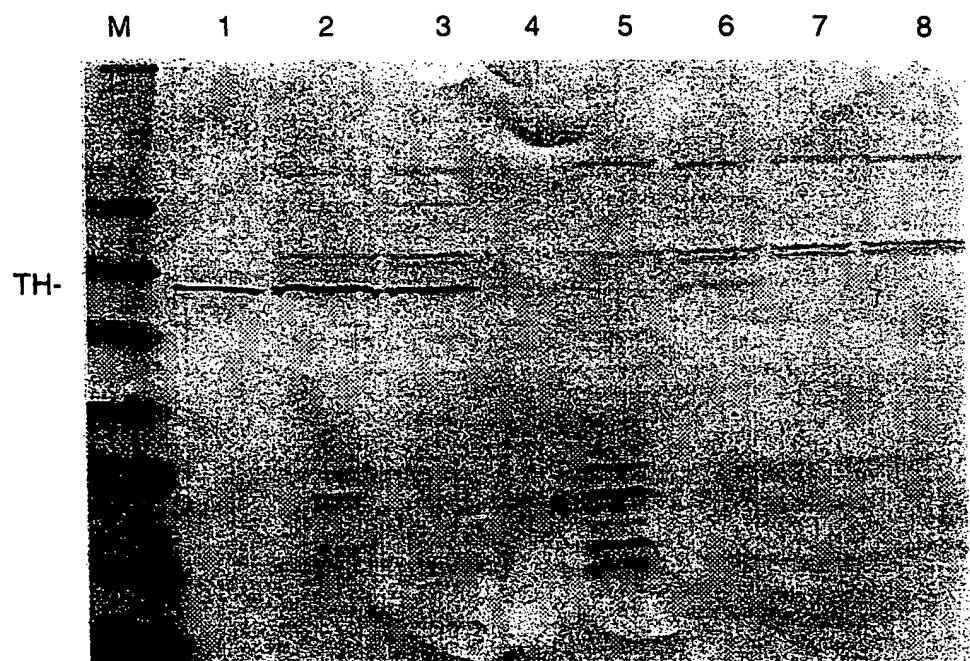
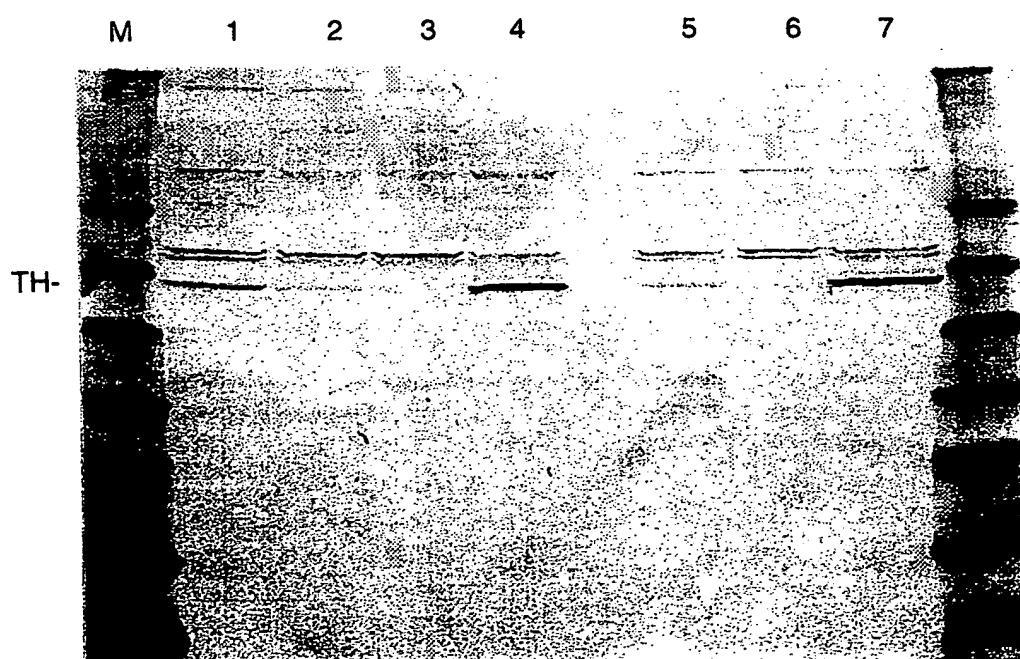
induce the production of bcl-2 production; and  
a pharmaceutical diluent.

16. The transplantable neuronal cells of claim 13, wherein the lithium salt is lithium chloride.

17. The method of claim 15, wherein the lithium salt is lithium chloride.

18. A chimeric non-human mammal wherein said mammal comprises post-mitotic dopaminergic neuronal cells implanted in the brain of said mammal.

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**FIG.\_1****FIG.\_2**

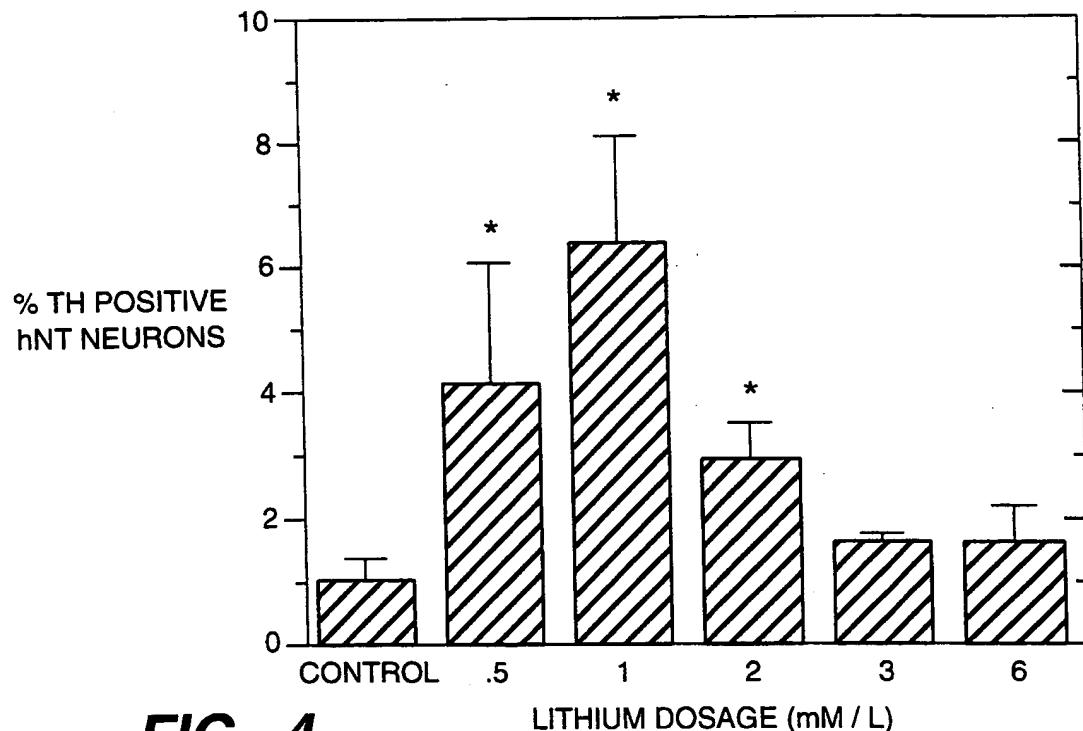
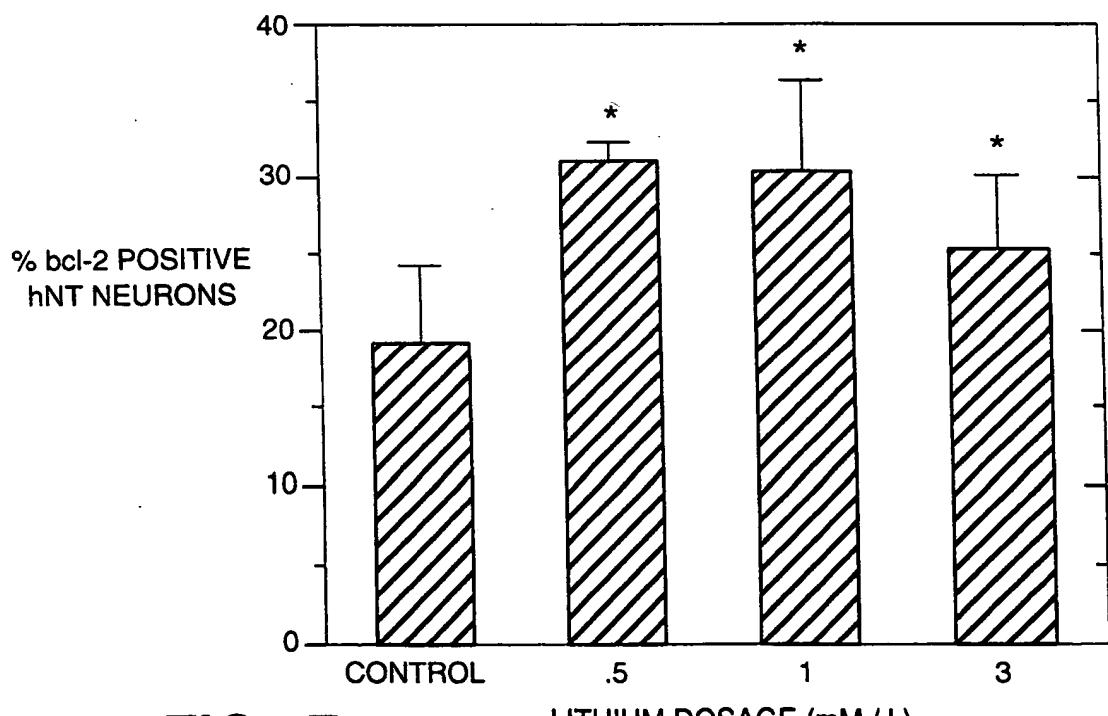
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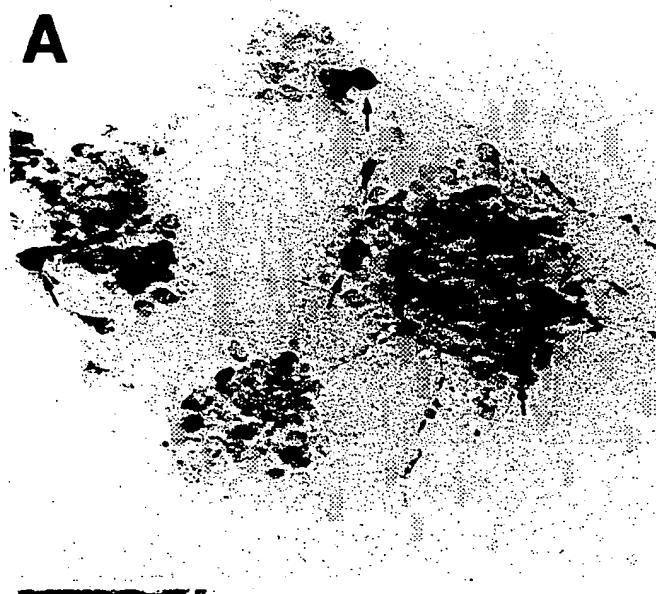
***FIG.\_3***

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**FIG.-4****FIG.-7**

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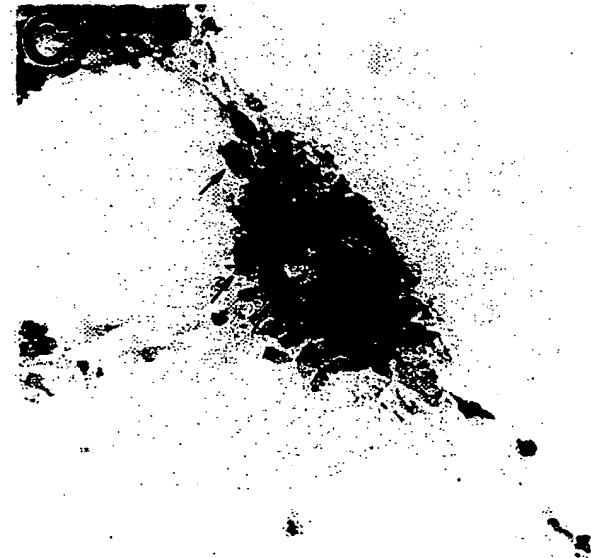
**FIG.\_5A**



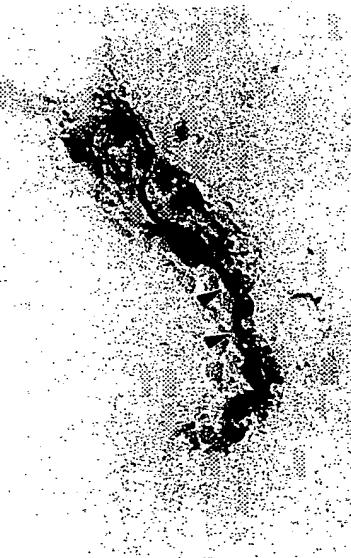
**FIG.\_5B**



**D**



**FIG.\_5C**



**FIG.\_5D**

A

**FIG.\_6A**

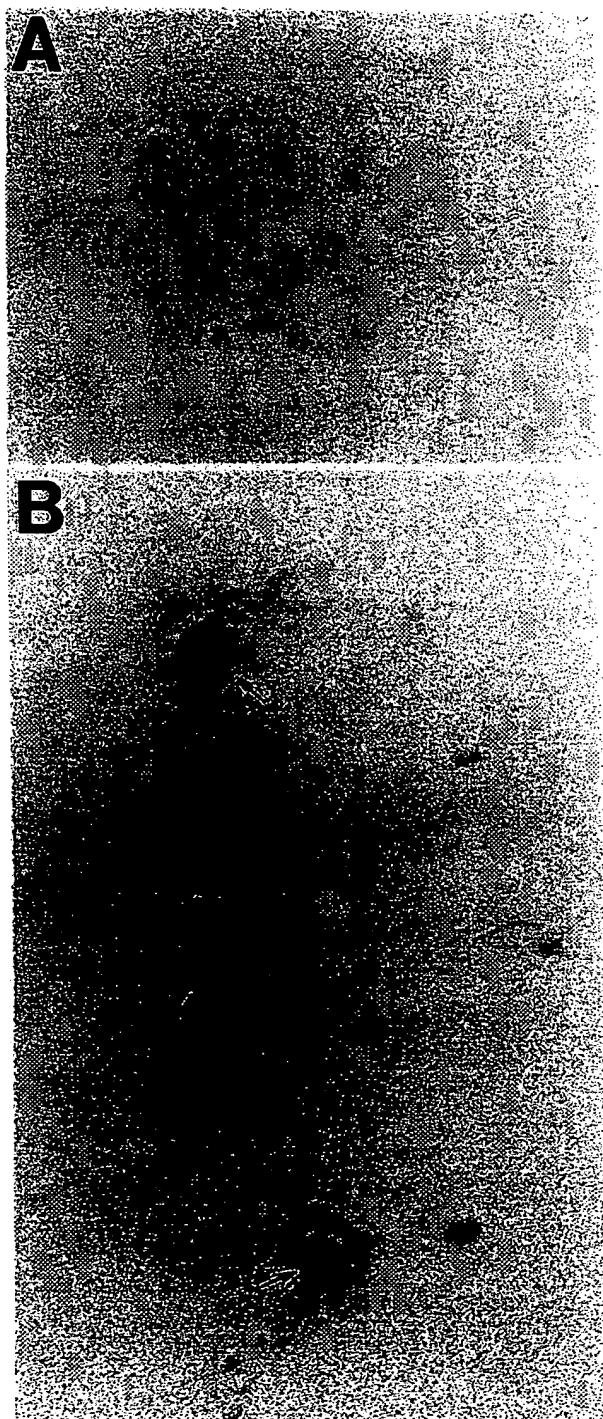
B

**FIG.\_6B**

C

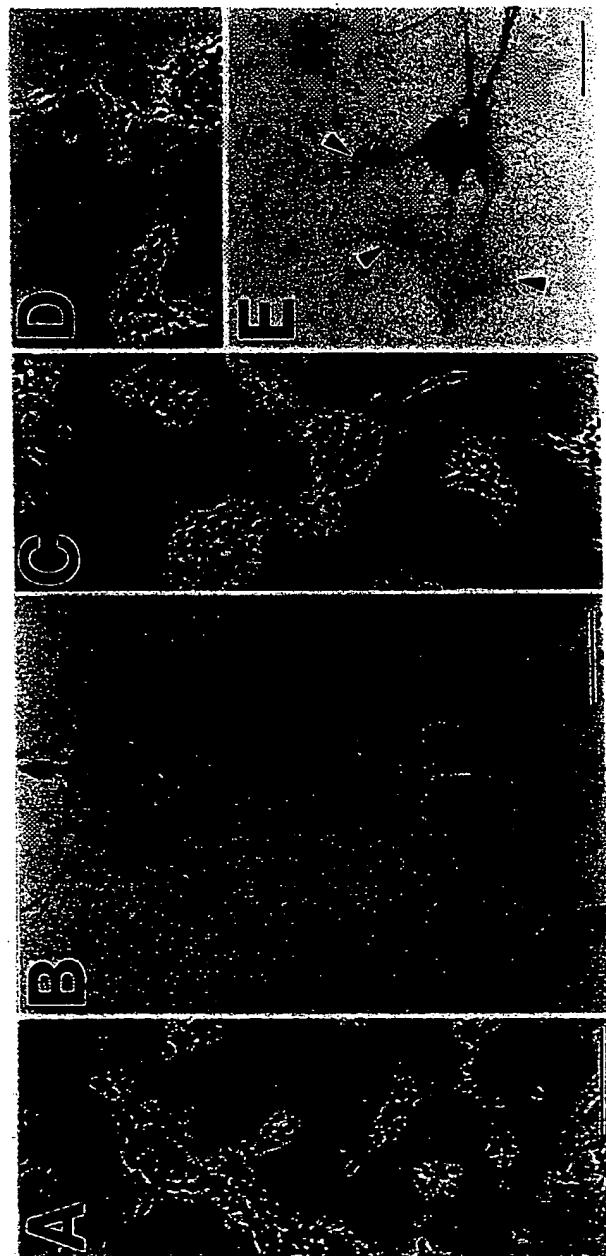
**FIG.\_6C**

***FIG.\_8A***



***FIG.\_8B***

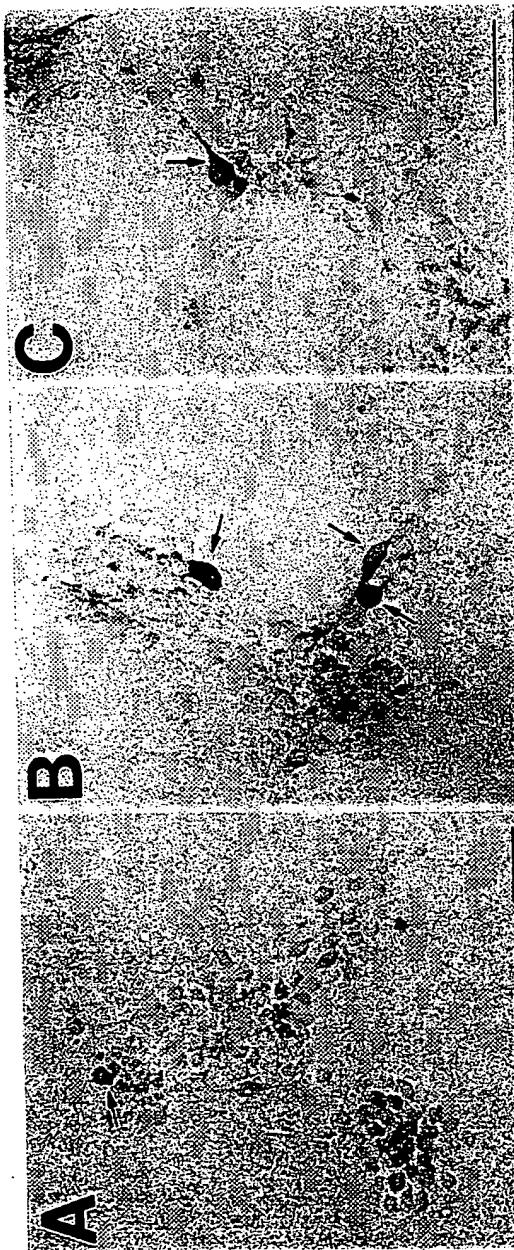
*FIG.\_9D*



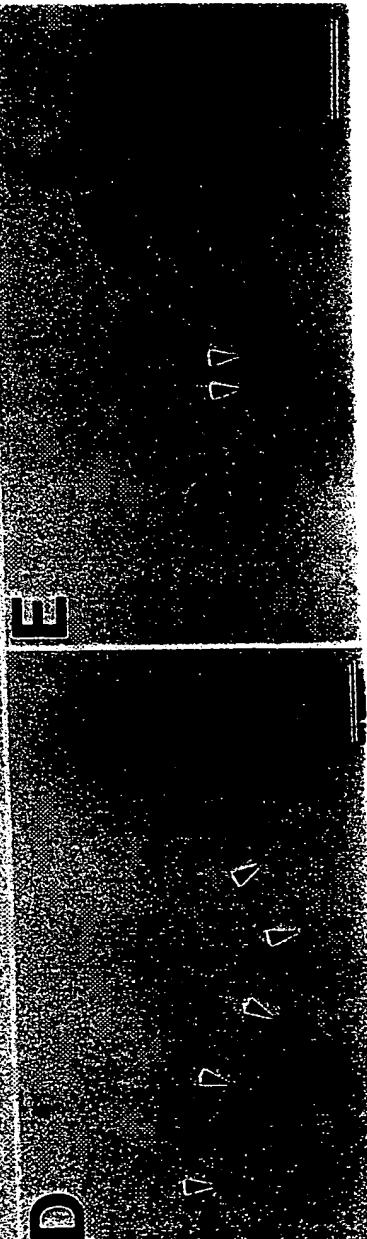
*FIG.\_9E*

*FIG.\_9A*    *FIG.\_9B*    *FIG.\_9C*

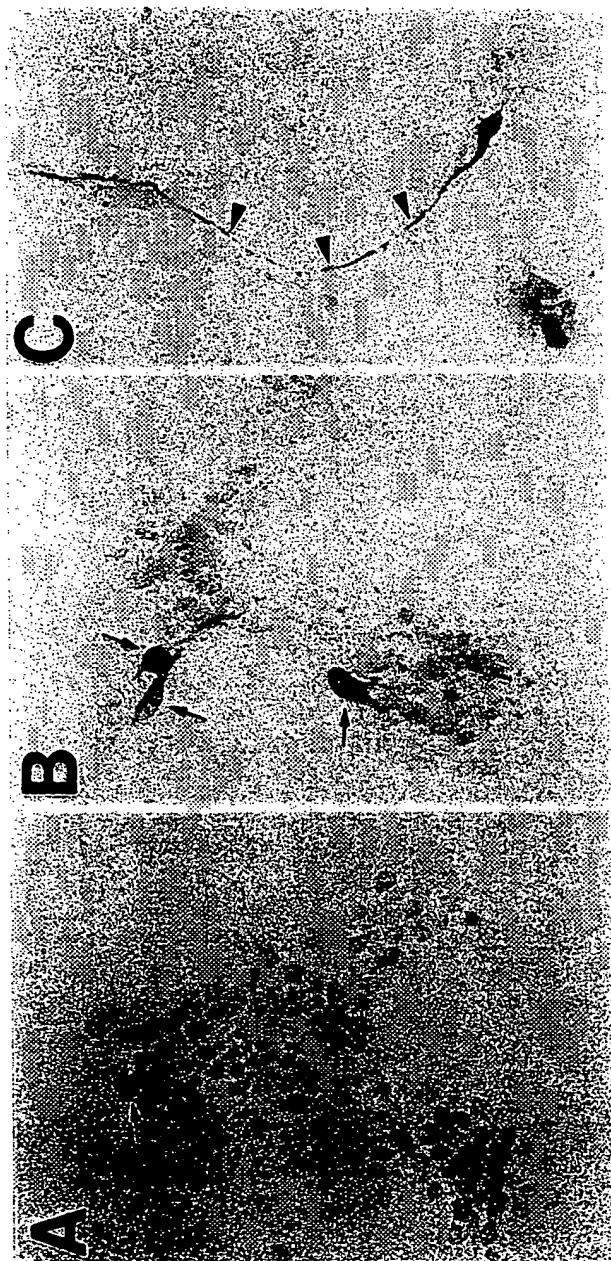
*FIG.\_ 10A      FIG.\_ 10B      FIG.\_ 10C*



*FIG.\_ 10D*



*FIG.\_ 10E*

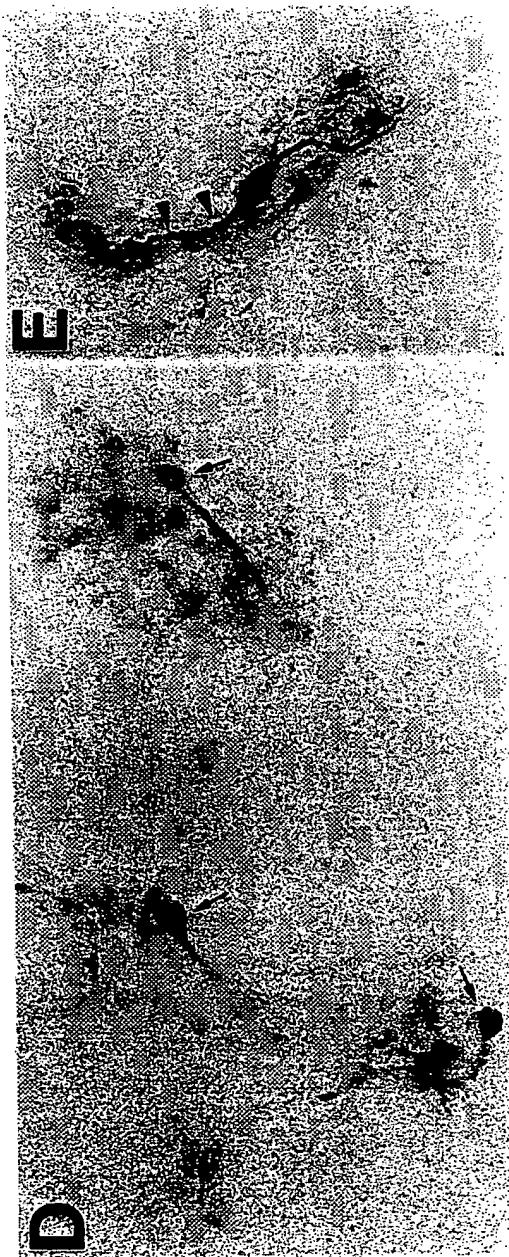


*FIG.\_ 11A*

*FIG.\_ 11B*

*FIG.\_ 11C*

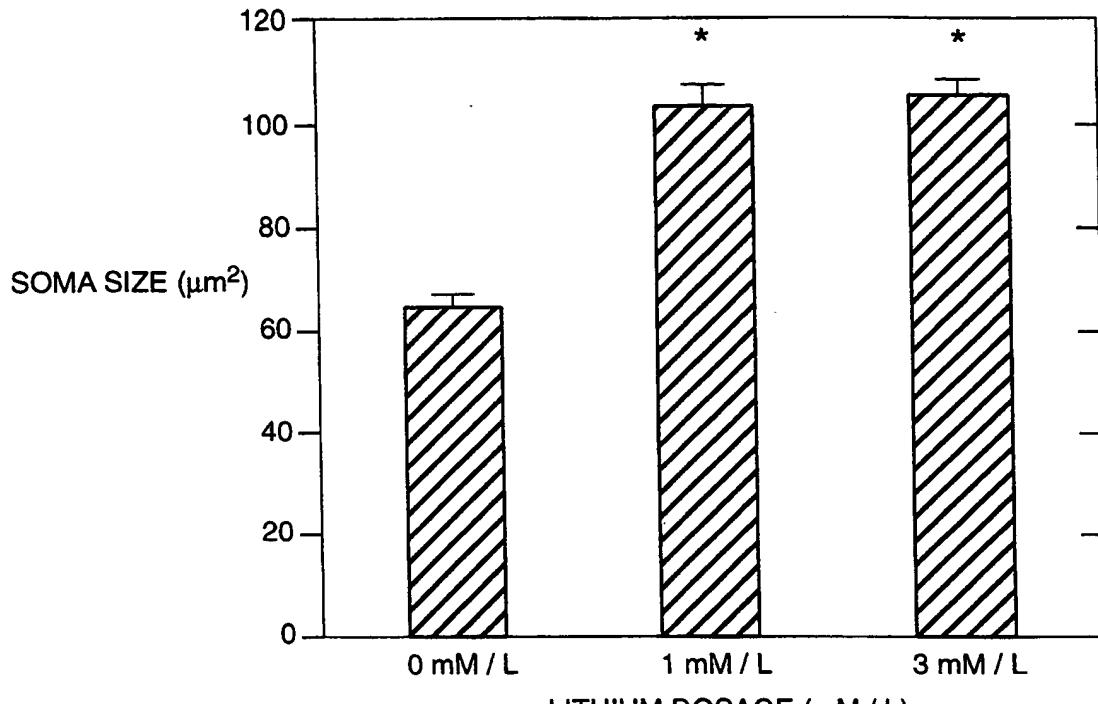
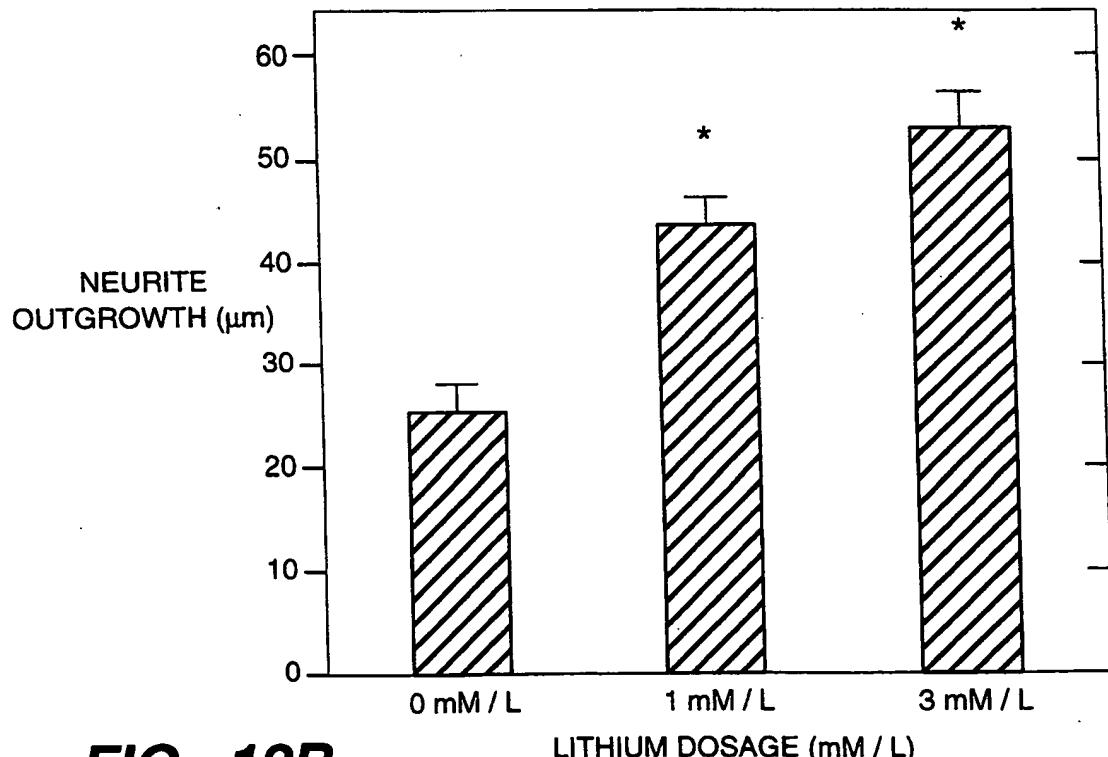
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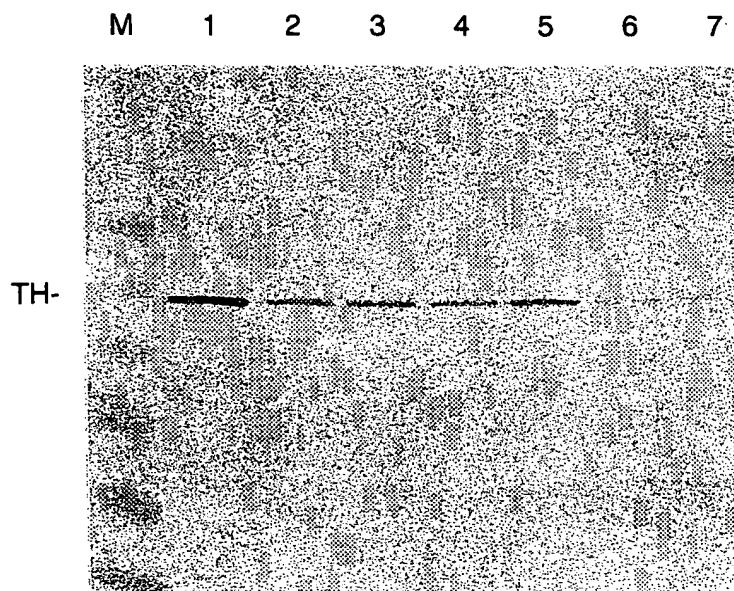
*FIG.\_ 11E*

*FIG.\_ 11D*

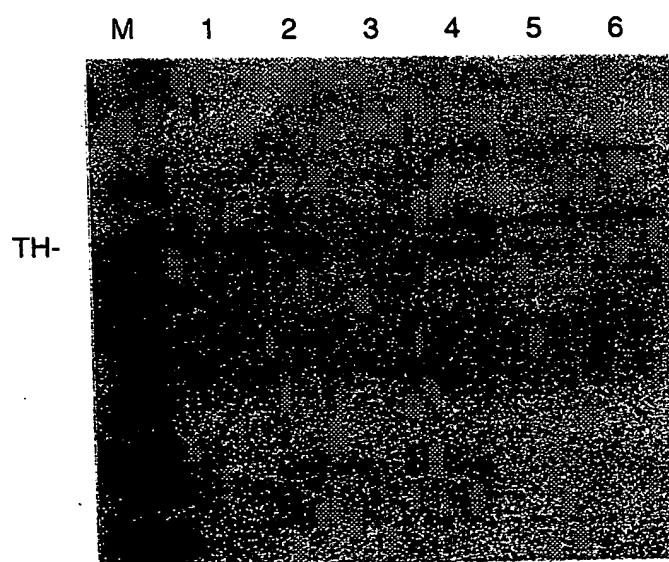
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**FIG.\_ 12A****FIG.\_ 12B**

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**FIG.\_13**



**FIG.\_14**

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/23977

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00; A01K 67/00; A01N 63/00  
US CL :435/325, 377; 424/9.2, 9.21; 800/8

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 377; 424/9.2, 9.21; 800/8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and Chemical Abstracts

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,175,103 A (LEE et al.) 29 December 1992, see entire document, especially col. 2, lines 56 to col. 3, line 2; col. 4, line 43 to col. 6, line 35 and col. 11, lines 22-32.	1-18
Y	SANDBERG, P.R. et al. Testis-Derived Sertoli Cells have a Trophic Effect on Dopamine Neurons and Alleviate Hemiparkinsonism in Rats. Nature Medicine. October 1997, Vol. 3, No. 10, pages 1129-1132, especially pages 1129-1131.	1-18

Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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*B* earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 FEBRUARY 1999

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PEKOSZ, A. et al. Induction of Apoptosis by La Crosse Virus Infection and Role of Neuronal Differentiation and Human bcl-2 Expression and its Prevention. Journal of Virology. August 1996, Vol. 70, No. 8, pages 5329-5335, see especially pages 5352-5334.	1-14
Y	LANGEVELD, C.H. et al. Cultured Rat Striatal and Cortical Astrocytes Protect Mesencephalic Dopaminergic Neurons Against Hydrogen Peroxide Toxicity Independent of their Effect on Neuronal Development. Neuroscience Letters. 1995, Vol. 192, pages 13-16, see especially pages 14 and 15.	1-14
Y	ADAMS, F. S. et al. Characterization and Transplantation of Two Neuronal Cell Lines with Dopaminergic Properties. Neurochemical Research, 1996, Vol. 21, No. 5, pages 619-627, see especially pages 621-626.	1-18